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MUSCLE MEMBRANES *in* DISEASES *of* MUSCLE

Robert E. Mrak

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Muscle Membranes in Diseases of Muscle

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PREFACE

My purpose in writing this book was twofold. The first objective was to provide a comprehensive, interdisciplinary review of research on membrane abnormalities in diseases of muscle. To this end there are nearly 800 literature citations in this book. The second objective was to organize this material in a logical and useful manner. This organization is threefold: by disease, by species (or model), and by discipline (or experimental technique).

The first chapter provides an overview of the techniques — physiological, ultrastructural, and biochemical — that have been used in the study of muscle membranes in diseased muscle. Each remaining chapter considers a human disease, or group of diseases, and their animal models. These models are just that — models — and not necessarily duplicates of a human disease in an animal. For this reason, research on each animal model is discussed separately, and conclusions reached within the context of this body of research before making extrapolations to and comparisons with the human disorders. For each human disease or animal model, experimental results are presented in a uniform manner, beginning with physiological studies (including muscle mechanics and electrophysiology), followed by ultrastructural studies and then biochemical studies of the function and composition of isolated muscle membranes. A summary is then provided in which interdisciplinary correlations are made.

Robert E. Mrak
Ferndale, Arkansas
November 29, 1984

THE EDITOR

Robert E. Mrak, M.D., Ph.D., is Assistant Professor of Pathology at the University of Arkansas, Medical Sciences Campus, and Chief of Electron Microscopy at the John L. McClellan Memorial Veterans Administration Hospital in Little Rock, Arkansas.

Dr. Mrak received his bachelor's degree in mathematics from the University of California at Davis in 1970. He received his predoctoral training at the same institution from 1970 to 1976 and was awarded his M.D. degree there in 1975 and his Ph.D. degree there in 1976. He served briefly as Instructor in Zoology in 1974. Dr. Mrak's thesis work, conducted in the muscle biophysics laboratory of Dr. Ronald Baskin, involved an ultrastructural and biochemical characterization of sarcoplasmic reticulum membranes isolated from mice with muscular dystrophy. Dr. Mrak's postdoctoral training was carried out at Vanderbilt University, in the departments of Pathology and Molecular Biology. Here, he was a resident in Anatomic Pathology (1976-1978) and a Muscular Dystrophy Association Postdoctoral Fellow in the membrane biochemistry laboratory of Dr. Sidney Fleischer (1978-1980).

Dr. Mrak served as Assistant Professor of Pathology in the Vanderbilt University School of Medicine (1980-1984) with clinical and teaching responsibilities involving the histological and ultrastructural diagnosis of neuromuscular disorders. During this time, he was awarded a Career Development Award from the Veterans Administration for work on muscle membranes in muscular dystrophy. Dr. Mrak is a member of the International Academy of Pathology and of the American Association of Neuropathologists.

Dr. Mrak's research has concerned the ultrastructure, function, and composition of muscle membranes in diseases of muscle. His primary interest has been in muscular dystrophy, but he has also authored or co-authored papers on alcoholic myopathy, muscle denervation, carnitine deficiency, acid maltase deficiency, and arthrogryposis.

DEDICATION

This book is dedicated to my parents, Dr. Vera Greaves Mrak and Dr. Emil M. Mrak.

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I. INTRODUCTION

A. Diseases of Muscle

Diseases of muscle are not prominent among the litany of mankind's ills, and they are different in several respects from disease elsewhere in the body. In contrast to diseases of other organ systems, infections and tumors of muscle are rare, and most of the important diseases of muscle are either hereditary or idiopathic. Diseases of muscle are different in the poverty and inadequacy of the resources available to the physician for the treatment or prevention of these diseases. They are different as well in the proven resistance of these diseases to elucidation of their basic pathogenetic mechanisms.

For most of the human diseases considered in this book, the etiologies are known: they are genetic diseases which are presumably the results of single mutations. And yet, despite the great advances of this century in the understanding of "inborn errors of metabolism", despite the elucidation of the elegant anatomic and biochemical basis of muscle function, despite the efforts of numerous laboratories and researchers, the pathogenetic steps involved in the expression of these diseases remain obscure. For these diseases, recent interest and research has focused on the membranous systems of muscle, and in particular on the three membrane systems involved in the process of initiating muscle contraction in response to a neural stimulus. This process is known as excitation-contraction coupling, and the three membrane systems involved are the muscle plasma membrane, the tubular invaginations of the plasma membrane known as the transverse tubule system, and the sarcoplasmic reticulum. The isolation and purification in vitro of these membranes is not a simple task, and the characterization of membrane-associated phenomena in general is technically more difficult than is the characterization of soluble enzymes and molecules. If the diseases considered in this book are indeed diseases of membranes, it is perhaps not surprising that our understanding of these genetic diseases of muscle has lagged behind our understanding of the genetic diseases of glycolysis, amino acid metabolism, and other metabolic pathways that do not involve membranes.

B. Muscle Membranes

Muscle contraction is accomplished through a system of sliding filaments, the elucidation of which was one of the major triumphs of early research in biological electron microscopy.[1] These filaments are "rowed" past one another by oar-like extensions (head groups) from one class of these filaments, the myosin filaments. The regulation of this process is accomplished along the surface of the other class of filaments, the actin filaments. Here, regulatory proteins (tropomyosin and troponin) hide the actin active sites in the presence of low (submicromolar) quantities of free calcium ions, so that the muscle is relaxed. At higher (micromolar) concentrations of free calcium ions, the actin active sites are exposed to the myosin head groups, and the muscle will contract.

It is the regulation of cytoplasmic levels of free calcium ions, then, that is the key to the control of muscular contraction. Calcium ions in muscle are actively sequestered in a membranous network known as the sarcoplasmic reticulum (Figure 1). This network, which is analogous to the endoplasmic reticulum in nonmuscle cells, has an ATP-driven calcium pump with a high affinity for calcium ions. The sarcoplasmic reticulum is intimately coupled to the muscle plasma membrane by means of an elaborate system of tubular infoldings of the muscle plasma membrane, known as the transverse tubule system. These tubular infoldings, which are arranged at frequent, regular intervals along the muscle fiber, run from the muscle fiber surface deep into the muscle fiber, and form junctional complexes with the sarcoplasmic reticulum. The

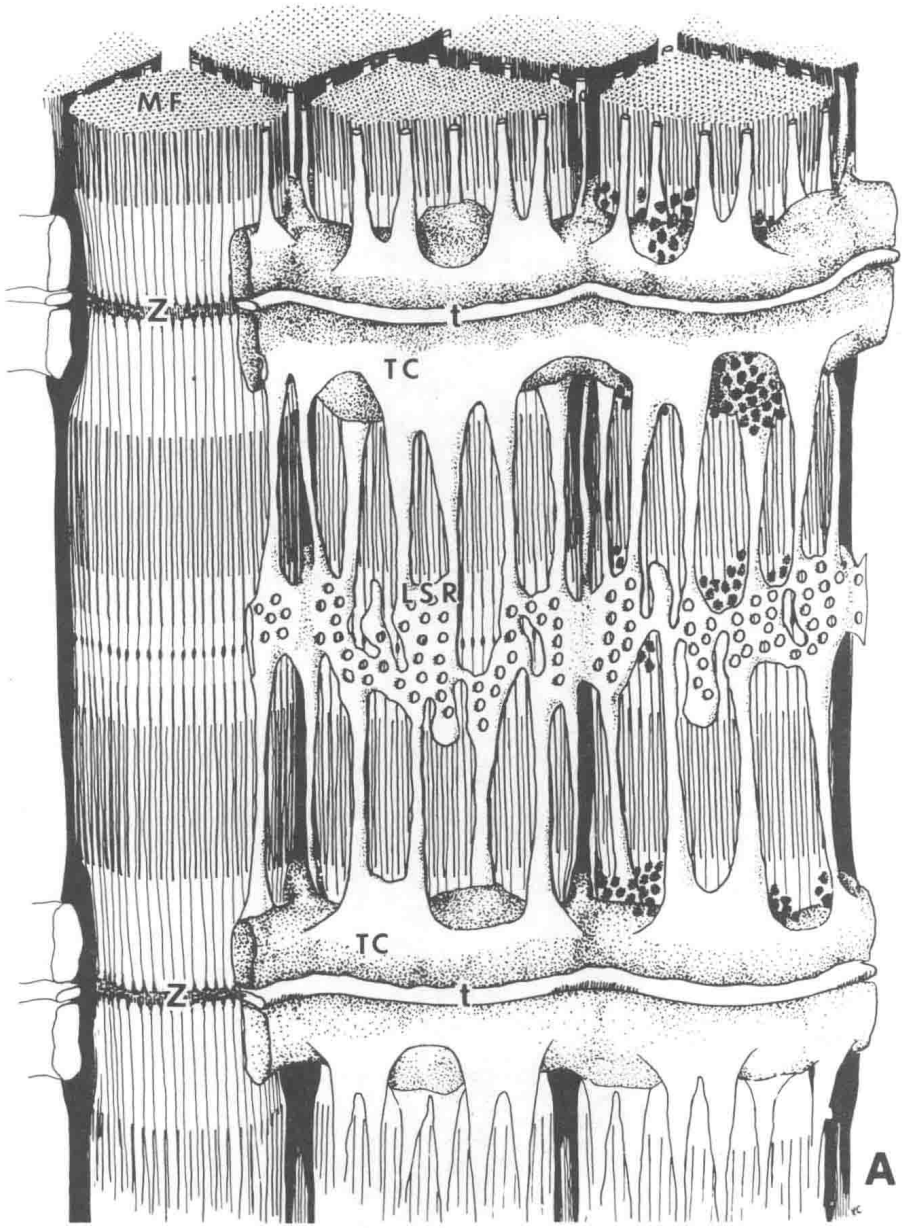

FIGURE 1. Artist's conception of the arrangement of muscle membrane systems within the muscle fiber. (A) Arrangement of muscle membranes in nonmammalian skeletal muscle. The transverse tubule system (t) crosses the muscle fiber perpendicular to the orientation of the myofilaments (MF), at the level of the Z-lines (Z). The sarcoplasmic reticulum consists of terminal cisternae (TC), which are located adjacent to the transverse tubules and form junctional complexes with them, and longitudinal elements (LSR), which run between adjacent terminal cisternae. (Reproduced from Peachey, L. D., *The Journal of Cell Biology*, 1965, vol. 25 (part 2), page 222, by copyright permission of the Rockefeller University Press.) (B) Arrangement of muscle membranes in mammalian skeletal muscle. The transverse tubules (t) are located at the junction of the muscle fiber A bands (A) and I bands (I), rather than at the Z lines (Z). There are consequently two transverse tubules per sarcomere, rather than one. Mitochondria (m) are also shown. SR = sarcoplasmic reticulum, M = M line. (Reproduced from Price, H. M. and Van de Velde, R. L., in *Disorders of Voluntary Muscle*, 4th ed., Walton, J. N., Ed., Churchill Livingstone, Edinburgh, 1981, page 51, by copyright permission of the publisher.)

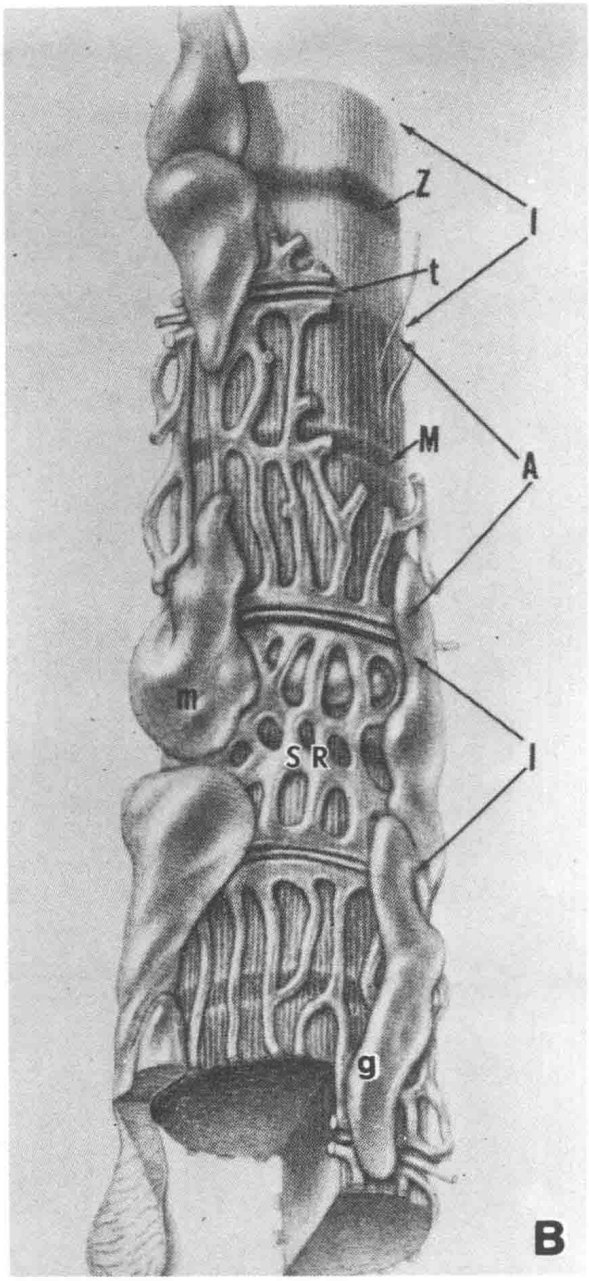

FIGURE 1B

lumen of the transverse tubule is consequently a continuation of the extracellular space. In conventional thin-section electron micrographs the combination of a transverse tubule and two associated membranous cisterns of the sarcoplasmic reticulum is known as a triad. In response to a neural stimulus, an action potential is propagated along the muscle plasma membrane and into the muscle interior by means of the transverse tubule system. There, a signal is passed across the transverse tubule-sarcoplasmic reticulum junction for the sarcoplasmic reticulum to release its stores of calcium, and muscle contraction commences. Following "recovery" of the sarcoplasmic reticulum from the "release" signal, the free calcium in the muscle cell is reaccumulated by the

sarcoplasmic reticulum, and the muscle relaxes. The structural nature of the junction between the transverse tubule and the sarcoplasmic reticulum, the biochemical mechanism of signal transfer between the transverse tubule and the sarcoplasmic reticulum, and the process of calcium release by the sarcoplasmic reticulum remain to be fully elucidated.

The sarcoplasmic reticulum can be differentiated into two distinct anatomic regions. The portions of the sarcoplasmic reticulum which are immediately adjacent to, and form triads with, the transverse tubules are known as terminal cisternae. In this area there is electron-dense proteinaceous material demonstrable within the lumen of the cisternae. Connecting one terminal cistern of the sarcoplasmic reticulum (adjacent to one transverse tubule) with a second terminal cistern (adjacent to the next transverse tubule) are tubular continuations of the sarcoplasmic reticulum known as longitudinal tubules, which do not have electron-dense proteinaceous material in their lumens. Unlike the lumen of the transverse tubule, the lumen of the sarcoplasmic reticulum does not connect with the extracellular space, and is thus a true intracellular compartment.

II. *IN SITU* TECHNIQUES

A. Physiological Techniques

Diseases of muscle are manifested clinically as alterations in muscle function, usually in the form of muscle weakness. It is in the study of such altered function — in the physiological investigation of diseased muscle — that the nature and extent of the alteration is precisely defined. Physiological studies of diseased muscle provide the framework within which questions as to the etiology and pathogenesis of the disease process must be formulated, and within which biochemical investigations of muscle membrane function must be pursued. Thus a review of the relevant physiological data, and of any involvement of muscle membranes implicated by such studies, is a necessary prelude to the pursuit of a possible membrane abnormality in a muscle disease.

1. Muscle Heat Production

Contracting muscle produces heat as a thermodynamic by-product of the metabolic and mechanical processes involved in muscle contraction. Both the extent and the pattern of this heat production are functions of the type and extent of activity in which the muscle is engaged. In an isotonic muscle contraction, the total energy liberated by the contracting muscle is the sum of work performed plus heat produced. In an isometric muscle contraction (in which no net work is performed), all of the energy liberated by the muscle is ultimately dissipated as heat. A. V. Hill published his now-classical studies of muscle heat production in 1938[2] defining shortening heat and relaxation heat, and elucidating the relationship of these to various contractile parameters. Several recent reviews of this field are available.[3-5] It is now thought that 30 to 50% of the energy liberated by contracting muscle is associated with calcium release and reaccumulation by the sarcoplasmic reticulum.[4] Thus, measurement of muscle heat production is a potentially powerful tool in investigating possible abnormalities of sarcoplasmic reticulum function in diseased muscle. Unfortunately, these measurements are technically difficult, even with normal muscle under optimal conditions, and only one study of muscle heat production in diseased muscle is known to this author.[6]

2. Muscle Mechanics

The physiological study of muscle shortening and force production is known as muscle mechanics. The simplest of these measurements are the determination of force production during an isometric twitch and an isometric tetanus, and these parameters are often found to be decreased in diseased muscle. However, degenerative changes in

muscle clearly will result in decreased ability of the muscle to generate force, and these measurements by themselves offer little insight into the pathogenesis of degenerative diseases. Of greater significance are measurements of contractile kinetics; the rate of rise of force, the time to peak tension, the rate of decay of force, and the time to relaxation. These parameters are dependent upon the excitation-contraction coupling mechanism of muscle (which involves the muscle plasma membrane, the transverse tubule, and the sarcoplasmic reticulum), and upon the relaxation mechanism of muscle (which involves the sarcoplasmic reticulum), as well as upon the contractile filaments. Abnormalities in contractile kinetics have been reported in various forms of human and animal muscular dystrophy, and these abnormalities suggest membrane alterations in these diseases.

Measurements of muscle mechanics have classically been performed in vitro using single muscles carefully dissected from the animal. The muscles chosen are small, to allow adequate oxygenation of the muscle in vitro. It is also possible to dissect from a muscle a single muscle fiber for these measurements, and experiments of this type minimize problems of interpretation arising from focal degenerative changes in diseased muscle. More recently, studies have appeared measuring muscle function in vivo, where the maintenance of normal blood flow assures adequate nutrition and oxygenation of the muscle under study. In humans, measurements have been performed by the noninvasive technique of attaching a force transducer to the thumb and measuring force production following stimulation of the ulnar nerve.

A limitation of standard measurements of muscle mechanics is the contribution of elastic elements, within the muscle itself, to the measured tension. During the rising phase of force production, the measured force will be less than the actual force being produced by the contractile apparatus because some of this strength is being used to stretch the muscle's own elastic elements, rather than to pull against the measuring device. Similarly, during the falling phase of force decay, the measured force will be greater than the actual force produced by the contractile apparatus because the stretched elastic elements of the muscle are shortening and pulling against the measuring device (the additional force "stored" in the elastic elements is being released). Only when total force produced by the muscle is not changing (during the plateau of a tetanic contraction, or during a peak of a twitch) will the measured force actually equal the force production of the contractile apparatus.

A series of "quick releases" can be used to measure the activity of the contractile apparatus itself (or the "active state" of muscle) following cessation of stimulation. In this type of study, the muscle is stimulated to contract isometrically. Then, after the stimulus has ended, the muscle is allowed to shorten a short distance, and is then once again held isometrically. The tension measured at the new isometric point will rise to a peak as the contractile apparatus of the muscle pulls once more against the elastic components of the muscle, and will then fall again with the falling activation. At the peak of redeveloped tension, the elastic elements of the muscle are neither shortening nor lengthening, and the tension measured is consequently that of the contractile elements alone. This allows determination of true active state tension at a specific time point during the falling phase of muscle activation. A series of such measurements allows determination of the kinetics of active state tension decay. Techniques are also available for measuring the duration of the active state plateau, or period of unchanging, maximum force production during a muscle twitch.[7] This plateau is not seen on standard tracings of muscle force production, because the elastic elements continue to lengthen and "absorb" energy until the plateau has passed. Experiments of this type have been performed on dystrophic mouse muscle.[8]

The electromechanical coupling time, or the interval between the appearance of an action potential at the muscle fiber surface and the initiation of contraction, can be

determined by the simultaneous recording of mechanical tension and plasma membrane electrical activity in muscle. Such a study in muscle from malignant hyperthermia-susceptible pigs has shown prolongation of this coupling time, suggesting an alteration in muscle excitation-contraction coupling in these animals.[9]

Isolated muscle fibers in vitro can be "skinned" of their plasma membrane, exposing the fiber contents to the bathing medium. This skinning can be accomplished mechanically, by disrupting the surface membrane,[10] or chemically.[11] Such preparations allow one to introduce ions and chemicals directly into the fiber interior, and to measure the mechanical force produced by the fiber in response to these ions or chemicals. The skinning technique has been used in the evaluation of biopsied muscle from patients with Duchenne muscular dystrophy.[12]

3. Electrophysiology

Muscle, like nerve, is an excitable tissue, and classical electrophysiological methods can be applied to the study of electrical phenomena in muscle, phenomena referable to the muscle surface membranes and the ionic gradients across these membranes. A muscle fiber is impaled with one or more microelectrodes, and the membrane potential is measured under different experimental conditions. A decreased resting membrane potential is frequently found in diseased muscle, but the significance of this is often obscure. Any damage to the muscle fiber, whether inflicted accidentally in the process of dissection or electrode insertion, or occurring naturally as the result of a degenerative process, will result in a lowered resting membrane potential that cannot be attributed to any specific membrane alteration. In addition, changes in the ionic content of the muscle fiber or of the extracellular fluid can alter the resting membrane potential. Such ionic changes can be quite difficult to document. Kerr and Sperelakis[13] found decreased average resting membrane potential in muscle fibers from dystrophic mice, but this decrease was entirely attributable to measurements performed on fibers showing ultrastructural signs of fiber degeneration. Ultrastructurally normal fibers had normal resting membrane potentials.

Measurements of action potential characteristics are more reliable indicators of membrane function than simple measurement of the resting membrane potential. Gruener et al.[14] found decreased resting membrane potentials in biopsies from patients with Duchenne muscular dystrophy, myotonic muscular dystrophy, myotonia congenita, and motor neuron disease. However in all of these diseases except motor neuron disease, the imposition of a normal membrane potential with a direct current injected through the stimulating electrode resulted in near-normal action potentials.

Muscle fiber resistance and capacitance can also be measured using electrophysiological techniques. These values, of course, depend on the surface area of the muscle fiber. The fiber capacitance, in particular, is many times the capacitance of nerve fibers, because of the existence of the transverse tubule system, and anatomic alterations in the transverse tubule system can result in changes in the muscle fiber capacitance. If the surface area of the fiber being studied is known, then the specific membrane resistance and specific membrane capacitance (or resistance and capacitance per unit surface area) can be calculated. Changes in these values may indicate alterations in the ionic permeability of the membrane itself. Measurements of muscle fiber resistance (or its reciprocal, muscle fiber conductance) in the presence of extracellular solutions of different ionic compositions allows one to evaluate the relative contribution of specific ionic conductances to the total membrane conductance.

B. Ultrastructural Techniques

The use of the electron microscope in the study of muscular disease offers several technical advantages over other disciplines, in particular the small amount of material

necessary and the relative ease of dealing with diseased tissue. Provided standard precautions are taken to ensure that a suitably small biopsy specimen is rapidly and adequately fixed, problems of interpretation arising from artifactual differences between normal and diseased specimens should be less troublesome than with functional investigations. Also, since the quantity of material obtained at biopsy is small, ultrastructural investigations are more readily performed on human material than studies requiring greater amounts of tissue. Unfortunately, the use of electron microscopy in the study of diseases of muscle has added disappointingly little to our understanding of these diseases. One area where ultrastructure does appear to have made a contribution is in muscular dystrophy, where morphometric analysis of dystrophic chicken muscle has revealed fine anatomic alterations that may explain some of the physiological alterations in this model,[15] and where electron microscopy of biopsied muscle from Duchenne patients has revealed focal discontinuities in the muscle plasma membrane that may represent an early step in muscle fiber degeneration in this disease.[16]

Preparative techniques for conventional thin-section electron microscopy will not be reviewed here, except to point out two special techniques for the identification of muscle membranes *in situ*. Tannic acid can be used to enhance ultrastructural detail of the sarcoplasmic reticulum membrane.[17] The use of this agent during fixation results in an intense, selective staining enhancement of the outer layer of the sarcoplasmic reticulum membrane, allowing ready identification of this organelle in routine electron micrographs. The transverse tubule, in contrast, can be distinguished by the incubation of the specimen, prior to fixation, with an electron-dense extracellular marker molecule, such as ferritin,[18] which will diffuse into the muscle fiber along the lumens of the transverse tubule system.

Another ultrastructural technique of great potential value in the study of muscle membranes is freeze-fracture electron microscopy (Figure 2). In this technique, a specimen is quick-frozen to −100°C, and then fractured in a vacuum. Such frozen specimens tend to fracture along the internal lipid-lipid interface of natural and artificial lipid bilayer membranes,[19,20] thus exposing the actual membrane interior. Also, since the fracture lines tend to follow natural membranes for extended distances, large *en face* views of these membranes are obtained which are difficult or impossible to achieve using the thin-section technique. The exposed fracture face is shadowed from an angle with vaporized electron-dense platinum, which builds up against any protrusions or irregularities in the fractured surface. This produces a shadow effect, and imparts a three-dimensional appearance to the final image. The shadowed fracture face is then given a second shadowing from above with vaporized, (relatively) electron-translucent carbon (to assure mechanical stability of the replica), and the finished platinum-carbon replica is then recovered, washed completely free of organic residue, and examined by standard transmission electron microscopy. Ice crystal formation during freezing is a major source of artifact in this technique, and this is usually minimized by briefly fixing the specimen in glutaraldehyde, and then incubating the specimen in a cryoprotectant such as glycerol prior to freezing. Several reviews of this technique are now available.[21-24]

III. IN VITRO TECHNIQUES

A. Isolation of Muscle Membranes

The definitive demonstration of a functional or compositional alteration in a muscle membrane requires the isolation of that membrane and the biochemical demonstration of the alteration in vitro. This is not an easy task. While proven techniques are available for the large-scale preparation of sarcoplasmic reticulum vesicles from the white muscle of rabbits, only rarely can these published techniques be directly applied to