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SUBCELLULAR EVENTS ASSOCIATED WITH THE DEVELOPMENT AND RELEASE OF ISOMETRIC TENSION IN POST-MORTEM BOVINE, RABBIT AND PORCINE SKELETAL MUSCLE

by

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INTRODUCTION

Almost all meat quality attributes are established during the first 24-48 hours after death when muscle undergoes a series of changes which eventually transform it into meat. For this reason, the biochemical and structural changes that occur in post-mortem muscle have been of interest to meats researchers for many years.

Tenderness is one of the quality factors that undergoes large changes during the development of rigor mortis. It has been widely supposed that muscle in a pre-rigor state is quite tender, but that it then becomes tough during the development of rigor mortis. It has recently been shown however, that this tenderness pattern occurs only if the muscle has been excised immediately after death and left free to shorten during the onset of rigor mortis. If the muscle is left attached to the skeleton and is therefore not free to shorten extensively, it is least tender immediately after death and becomes progressively more tender with increasing time post-mortem. The causes of these differences in post-mortem tenderness changes have remained obscure.

Recently, it has been shown that post-mortem muscle held isometrically, will first develop tension, and then after varying periods of post-mortem time, gradually lose the ability to maintain this isometric tension. At low storage temperatures, loss of the ability of a muscle strip to maintain isometric tension occurs at about the same time that tenderness in that muscle begins to increase. In spite of extensive characterization of the biochemical changes that occur in post-mortem muscle, this

loss in ability to maintain isometric tension is the first physical property of post-mortem muscle that has been found to be related to the increase in tenderness post-mortem. Consequently, it seemed probable that further studies of the development and decline of isometric tension in post-mortem muscle would help clarify the nature of the events which cause changes in tenderness during the first 72-96 hours post-mortem.

The experimental approach adopted in this study was to use fiber strips or myofibrils made immediately after death, and then to place these strips or myofibrils in a controlled environment. This made it possible to study the effect of only one or two variables at a time on post-mortem isometric tension development or myofibrillar structure. Thus, initial experiments were designed to study the effect of storage at various pH levels on the isometric tension development of muscle strips and the adenosinetriphosphatase activities of myofibrils to ascertain whether pH per se was important in post-mortem changes in tenderness. Since a very large number of variables were involved and since it seemed desirable to conduct these studies at several different storage temperatures, it was necessary to limit the scope of the experiments. Hence, most experiments were designed to study loss of the ability to maintain isometric tension. This choice was made because it seems likely that this loss is related to the increased tenderness caused by post-mortem aging of meat. The results of a number of studies have indicated that connective tissue does not exhibit any large biochemical or ultrastructural changes during the first 96 hours post-mortem. Therefore, it appears that most of the changes in

tenderness during the onset and resolution of rigor mortis are a consequence of changes in the myofibrillar proteins and their interactions. Thus, the experiments in this study were directed specifically toward understanding the nature of post-mortem changes in the ultrastructure of the myofibril.

The study of post-mortem changes in the myofibrillar protein is of interest for reasons other than understanding meat tenderness. It has been shown that post-mortem shortening is accompanied by a sliding of filaments and by changes in the cross-striated banding pattern of striated muscle identical to those that occur during in vivo contraction. However, post-mortem shortening occurs over a period of several hours in contrast to the period of milliseconds required for in vivo contraction. Thus, muscle samples can easily be removed for study at intermediate stages of contraction. The fact that post-mortem shortening is temperature dependent offers further control over the process. Consequently, post-mortem shortening may be a valuable model system for the study of muscle contraction as well as an important process in post-mortem tenderization.

Abbreviations

The following abbreviations will be used throughout this thesis: Ca^{++}, calcium; Mg^{++}, magnesium; g, grams; ATP, adenosinetriphosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, 1,2-bis(2-dicarboxymethylaminoethoxy)-ethane; Tris, tris-(hydroxymethyl)-aminomethane; hr, hour; mM, millimolar; DOC, deoxycholate; CP, Creatine phosphate; SDS, Sodium dodecyl sulphate.

LITERATURE REVIEW

The physical and chemical changes occurring in post-mortem muscle have been studied extensively during the past three decades. Most of the early work on rigor mortis emanated during the 1930's from the Low Temperature Research Station at Cambridge, England. There, E. C. Bate-Smith, J. R. Bendall, and co-workers discovered that muscle became inextensible during the onset of rigor mortis and developed a quantitative measure of rigor based on this loss of extensibility. Moreover, many of the chemical changes which occur in post-mortem muscle were first described by the Cambridge group. Of necessity, this early work on rigor mortis was based on, and also contributed to the early understanding of muscle biochemistry. In the late 1930's, A. Szent-Gyorgyi and co-workers in Hungary made a number of discoveries which formed the basis for most of the modern research in muscle biochemistry. During this period in Hungary, the discovery of actin was described, and many of the techniques still in use for preparation of myofibrillar proteins were developed. Szent-Gyorgyi's findings were not widely publicized until after World War II, and his reports were soon followed in 1953, by Huxley and Hanson's discovery of the thick and thin filament structure of the myofibril. Szent-Gyorgyi's and Huxley and Hanson's findings have such fundamental importance to muscle biochemistry that a thorough acquaintance with them is now a necessary prerequisite to any meaningful discussion or rigor mortis. Consequently, this review will be divided into three sections. The first section will deal with the existing concepts of muscle action in

terms of Hanson and Huxley's thick and thin filament structure of muscle. This section is then followed by a review of subcellular events associated with rigor mortis. Finally, a short section on the properties of red and white muscle is included at the end of this review.

Muscle Biology

Myofibrils consist of two major proteins, myosin and actin, which by themselves are both necessary and sufficient for in vitro contraction. However, contraction of purified actin and myosin proceeds until ATP supplies are exhausted, and it is not possible to initiate or stop the contraction of purified actin and myosin in the presence of ATP. Hence, some method must be provided for control of the actin-myosin contraction. In 1946, Bailey (1946) discovered a third protein, tropomyosin, in the myofibril, and in the past four years, three other myofibrillar proteins have been described. These proteins have been named troponin, α-actinin and β-actinin. It now appears that these four additional proteins act to control or regulate the actin-myosin interaction in a way that makes it possible to initiate or stop the contractile process in the presence of ATP. Consequently, these four proteins have been termed regulatory proteins. The nature of their effect on the actin-myosin interaction will be discussed in more detail later.

In 1953, Hanson and Huxley (Huxley, 1953; Huxley and Hanson, 1953) first proposed that myofilaments making up the myofibril were in fact composed of two structurally different kinds of filaments arranged in an interdigitating array. These two kinds of filaments were named simply

thick and thin filaments. The thick filaments are cylindrical tubes approximately 1600A° long and about 150A° in diameter near their center, but tapering gradually toward both ends. In cross section, the thick filaments form a regular hexagonal array in the A-band. A number of lines of evidence indicate that myosin is found only in the thick filaments (Hanson and Huxley, 1955; Corsi and Perry, 1958; Corsi et al., 1967) and Huxley's (1963) demonstration that "synthetic" thick filaments, structurally very similar to natural thick filaments, could be produced by proper adjustment of the pH and ionic strength of purified myosin solutions, suggests that the thick filaments must be composed almost entirely of myosin. Thick filaments are held in the proper three dimensional array by protein cross-bridges joining them at their centers. When muscle is viewed in appropriate cross-section, the presence of these protein cross-bridges gives the structural entity called the M-line.

Huxley (1963) first proposed a model whereby myosin molecules were packed together to form thick filaments, and this model has recently been extended in several elegant papers by Pepe (1967a, 1967b). A number of physicochemical studies have shown that the myosin molecule is shaped like a rod with a diameter of approximately 15A° and a length of 1600A°. One end of the rod is enlarged to form a globular head about 40A° in diameter and extends about 250A° of the 1600A° total length of the molecule. Very brief treatment with trypsin splits this rod-shaped molecule near its center to form two fragments. The larger fragment containing the head is called heavy meromyosin whereas the thinner or tail fragment is called

light meromyosin. It has been shown that all of the ATPase activity and actin-combining ability of the original myosin molecule is located exclusively in the heavy meromyosin part of the molecule. Furthermore, Huxley (1963) has shown that it is possible to produce synthetic thick filaments by incubation of purified light meromyosin solutions at the proper pH and ionic strength. These light meromyosin filaments are structurally similar to natural or native thick filaments except that they do not possess any projections or cross-bridges extending from their surface. This and other lines of evidence (Pepe, 1967b) suggest that the light meromyosin part of myosin forms the shaft of the thick filament and that the heavy meromyosin or head part of myosin makes up the cross-bridges observed on the surface of the thick filament (Huxley, 1957).

Actin has been shown to be situated only in the thin filaments (Hanson and Huxley, 1953, 1955), and electron micrographs of negatively stained (Hanson and Lowy, 1963, 1964) or replicated preparations (Depue and Rice, 1965) have shown that "natural" thin filaments are structurally very similar to filaments of purified F-actin. Both are approximately 80A° in diameter and consist of two chains of globular subunits twisted around each other in a right-handed helix. The crossover points of the double-helical array, i.e., the pitch of the helix, is about 350A°, and there are 13 subunits in the two chains between each crossover point.

The globular subunits are about 55A° in diameter and presumably represent G-actin monomers. Native thin filaments from rabbit muscle are uniformly 10,000A° long, but the length of F-actin filaments varies widely

with an average of 80,000A° (Hama et al., 1965). Maruyama and co-workers (Maruyama, 1966a; 1966b) have recently suggested that the presence of β-actinin is responsible for controlling the length of natural actin filaments at 10,000A°, but neither the properties nor the mechanism of action of β-actinin is known.

Although actin appears to be located exclusively in the thin filament, the thin filament is not composed of actin alone. Several studies (Corsi et al., 1967; Endo et al., 1966; Perry and Corsi, 1958) have shown that tropomyosin is also located in the thin filament, and recently, Ebashi and co-workers (Endo et al., 1966; Ohtsuki et al., 1967) have presented evidence that troponin is distributed along the length of the thin filament with a periodicity of 400A°. Since neither troponin nor tropomyosin were seen in Hanson and Lowy's (1963) micrographs of negatively stained thin filaments, the exact distribution of actin, tropomyosin and troponin in the thin filament is presently unclear. However, several biochemical studies have shown that tropomyosin interacts strongly with F-actin (Ebashi, 1963; Ebashi et al., 1968) and furthermore, that tropomyosin interacts strongly with troponin (Ebashi and Kodama, 1965). Hence, it is currently felt that tropomyosin may serve as a "cement" to bind troponin to the actin filament.

Thin filaments are held in the proper three dimensional array by the Z-line. In electron micrographs of muscle, the Z-line appears as a heavily stained, dark band oriented transversely to the long axis of the myofibril. One end of the thin filament is embedded in the Z-line, and the thin filaments then extend out toward the center of the sarcomere,

terminating at the edge of the H-zone. Huxley (1963) has shown that the thin filaments are polarized and "point" in opposite directions on either side of the H-zone. The protein composition of the Z-line is unknown. Early studies suggested, mostly on the basis of indirect or inconclusive evidence, that tropomyosin was situated in the Z-line. However, Stromer and co-workers (Stromer et al., 1969) found that tropomyosin would not reconstitute the Z-line structure in Z-line extracted myofibrils, and furthermore, that it was possible to reconstitute the Z-line by using protein solutions which did not contain tropomyosin. Recently, Goll et al. (1967; 1969) have suggested that α-actinin is located in or next to the Z-line. However, there does not appear to be enough α-actinin in muscle (Robson, 1969) to account for the 5-6% of myofibrillar protein which Huxley and Hanson (1957) suggest as the Z-line composition of muscle.

According to the sliding filament theory of muscle contraction (Huxley and Hanson, 1954, 1960; A. F. Huxley and Niedergerke, 1954), length of the thick and thin filaments remains constant during contraction, and shortening is accompanied by a sliding together of the interdigitating filament array. In this model, only the cross-bridges change in conformation. It is presumed that the cross-bridges undergo a repetitive cycle of first interacting with a specific site on the actin filament, then having this interaction broken, and interacting with a second site along the thin filament. The interaction between the cross-bridge and the actin filament apparently creates a directional force pulling the actin filament in

toward the center of the sarcomere so that the second cross-bridge-actin-filament interaction occurs at a site on the actin filament which is closer to the Z-line than the first site was.

An overwhelming amount of evidence supporting the sliding filament hypothesis of contraction has accumulated. X-ray diffraction patterns of muscle show that proteins in the thick and thin filaments do not undergo any major structural change during contraction (Elliott et al., 1967; Huxley and Brown, 1967), a finding consistent with the sliding filament hypothesis. The only observable change in X-ray diffraction pattern of muscle during contraction is a slight decrease in intensity of a series of layer lines at orders of 429A^{o} (Huxley and Brown, 1967). These layer lines have been ascribed to the projections on the thick filament, and a decrease in their intensity during tension development suggests that they may be moving out of phase and thus fail to give an ordered X-ray diffraction diagram. This is clearly consistent with the sliding filament hypothesis of muscle contraction. In addition to these X-ray diffraction results, numerous light and electron microscopy studies have shown that during contraction, the I-band shortens and the H-zone narrows or disappears. These changes in banding pattern during contraction are identical to those that are predicted by the sliding filament theory. Furthermore, the banding pattern of severely contracted ("supercontracted") muscle can be most easily explained in terms of the sliding filament theory (Stromer et al., 1967) . Because of this evidence, it is now generally felt that muscle shortening must proceed via a sliding of filaments.

Although actin and myosin are the only proteins required for muscle contraction, a number of studies have shown that Mg^{++} and ATP must also be present for the contractile process to occur. Mg^{++} apparently acts to bind ATP to the myosin molecule (specifically the heavy meromyosin part of the myosin molecule), and then in the presence of actin, myosin splits the terminal phosphate off the ATP molecule, thereby providing energy for contraction. The manner in which ATP splitting is coupled to tension development by cross-bridges remains unknown. Although the actin-myosin-ATP-Mg^{++} system is capable of producing an *in vitro* contractile response, the contraction cannot be reversibly stopped and started, as must be done in the living organism. In the past four to five years, Ebashi (1963) and co-workers (Ebashi *et al.*, 1964, 1967) have shown that although purified actin and myosin will give a contractile response in the presence of Mg^{++} and ATP and in the absence (10^{-8}M or less) of Ca^{++}, the actin-myosin-tropomyosin-troponin complex will produce a contractile response only when trace amounts (10^{-6} to 10^{-5}M) amounts of Ca^{++} are present in addition to Mg^{++} and ATP. A great deal of recent work on this discovery has shown that troponin binds Ca^{++} very strongly (Ebashi *et al.*, 1967) and that binding of Ca^{++} causes conformational changes in the troponin molecule (Ebashi *et al.*, 1968). Other studies have shown that muscle contraction *in vivo* can be initiated and stopped by simply regulating the levels of intracellular free Ca^{++}. Thus, it appears that muscle contraction is initiated by a rise in intracellular free Ca^{++} levels. When the free Ca^{++} concentration is again lowered to 10^{-8}M or less, the actin-myosin interaction is terminated and relaxation occurs.

Numerous studies have shown that thick and thin filaments are pulled apart very easily in resting or relaxed muscle (A. Szent-Gyorgyi, 1947; H. H. Weber, 1960). Evidently, the actin-myosin interaction is prevented in some way in resting muscle. Considerable evidence has now accumulated that suggests that the Mg^{++}-ATP complex is capable of breaking or preventing the actin-myosin interaction. Thus, ATP has a dual role in muscle contraction. On the one hand, in the presence of Mg^{++} and Ca^{++}, its terminal phosphate is split off to produce energy for shortening. On the other hand, in the presence of Mg^{++} but in the absence of Ca^{++}, it prevents the actin-myosin interaction and causes relaxation. High levels (10^{-2}M) of Mg^{++} favor complete binding of ATP to the myosin filament, and this will produce dissociation of the actin-myosin complex, especially if Ca^{++} is absent. In the presence of low levels ($2x10^{-6}$M) of Mg^{++}, very little of the ATP is bound to myosin and contraction is favored, even in the presence of low Ca^{++} levels. Since distilled water and many chemical reagents contain 10^{-6} - 10^{-7}M Ca^{++}, chelating agents which bind Ca^{++} but not Mg^{++} often must be added to <u>in vitro</u> solutions in order to lower Ca^{++} levels to a point (10^{-7} - 10^{-8}M) where contraction will cease (Weber and Winicur, 1961). It was this contamination by low levels of Ca^{++} that delayed the discovery that muscle contraction is initiated and stopped by intracellular regulation of free Ca^{++} levels.

Concomitantly with the recognition that muscle contraction was regulated by Ca^{++}, a subcellular fraction which actively accumulated Ca^{++} was isolated by differential centrifugation of muscle homogenates (Baltscheffsky,

1964). The isolation of this subcellular fraction was presaged by the discovery that the fiber volume of whole muscle homogenates would undergo a remarkable decrease after several hours post-mortem (Marsh, 1951, 1952a; and Bendall, 1953). 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 syneresis of myofibrils. It was discovered that the response to added ATP was governed by a sarcoplasmic factor, and soon afterward, it was shown that this sarcoplasmic factor was particulate in nature and could be sedimented from muscle homogenates (Ebashi, 1960, 1961). In the presence of the sarcoplasmic factor, ATP addition caused an increase in the fiber volume, but in the absence of the sarcoplasmic factor, ATP addition caused only syneresis. Since syneresis of myofibrils is related to muscle contraction, whereas an increase in fiber volume or swelling of myofibrils is related to muscle relaxation, the sarcoplasmic factor soon became known as the "relaxing factor".

Electron microscopic examination of the particulate relaxing factor after it had been sedimented from muscle homogenates revealed that it consisted of vesicular and tubular membranes. When Ebashi and Lipman (1962) found some complete triad structures in electron micrographs of relaxing factor preparations, it was suggested that the relaxing factor, which had been identified because of its biochemical effects on myofibrils, was identical with the sarcoplasmic reticulum, a membranous structural entity seen in electron micrographs of muscle cells. This work together with

other ultrastructural studies (Franzini-Armstrong and Porter, 1964; Hasselbach, 1968) showed that the sarcoplasmic reticulum consists of a fine network of membrane-limited tubules interlaced among the myofibrils. This network of tubules can be divided into two anatomically and physiologically different 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 in toward the center of the fiber at regular intervals along the length of the fiber. These tubules, oriented at right angles to the long axis of the fiber, have been shown to be invaginations of the sarcolemma into the center of the cell (Huxley, 1964). Therefore, the lumen of the T-tubules communicates directly with the extracellular space. The L- or longitudinal system consists of a series of smaller tubules extending in either direction from the T-tubules and enveloping the myofibrils as lace-like sleeves. As these smaller tubules approach the larger T-tubules, they join to form large lateral cisternae adjacent to either side of the T-tubule. When sectioned in the proper plane and examined in the electron microscope, a T-tubule together with its two associated lateral cisternae on either side appear as three membrane-limited lumens. This structure has been called a triad. The T-tubules do not communicate with the lumen of the lateral cisternae, and the T- and L-systems are separate sets of membrane-enclosed tubules.

A number of studies have shown that T-tubules function to transmit the nerve impulse from the sarcolemma inward to the center of the cell

(Constantin and Podolsky, 1967; Huxley and Taylor, 1958). Since T-tubules occur regularly along the length of the fiber with a frequency of one or two per sarcomere, they provide a very thorough and efficient means for rapidly transmitting the nerve signals to all parts of the muscle cell. On the other hand, several electron microscopic studies, using precipitation of electron-dense calcium oxalate (Constantin et al., 1965; Ishikawa, 1968; Peachey and Schild, 1968) or autoradiography (Winegrad, 1965) as a means for detecting areas of Ca^{++} localization, have shown that the longitudinal system is the Ca^{++}-binding system in muscle.

Although it is now possible to associate the T- and L-systems of the sarcoplasmic reticulum with specific biochemical functions, this still provides no insight into the mechanism whereby Ca^{++} is bound and released. Since it is obviously difficult to study the mechanism of Ca^{++}-binding by the sarcoplasmic reticulum in intact muscle cells, most studies on mechanisms of Ca^{++} sequestration have been done on vesicular preparations made by differential centrifugation of muscle homogenates. Studies on these preparations have shown that they actively accumulate Ca^{++} against a concentration gradient and that they are able to lower free Ca^{++}-concentration to 10^{-7}M or less, a level low enough to cause relaxation of myofibrils. This Ca^{++}-accumulation is coupled to a Mg^{++}-activated ATPase activity possessed by the vesicular preparations. ATP is essential for Ca^{++}-accumulation and cannot be replaced by other nucleotide triphosphates (Baird and Perry, 1960). Although a small amount of Mg^{++}-modified ATPase activity is exhibited by vesicular preparations at very low (10^{-8}M) Ca^{++} concentration, this activity is increased seven to eight-fold by increasing

the Ca^{++} level to 10^{-5}M (Hasselbach and Makinose, 1962). The addition of oxalate to in vitro assays of vesicular ATPase activity increased the Ca^{++} uptake by the vesicules several fold (Hasselbach, 1964), presumably because the accumulated Ca^{++} is precipitated inside the membrane-enclosed vesicles as calcium oxalate and is thereby not free to escape to the external medium. Aging of the vesicular preparations causes a rapid decrease in Ca^{++} sequestering ability (Baird and Perry, 1960), probably due to lability of the membranes during storage. On the basis of histochemical studies, Gauthier (1967) has suggested that the L-system may possess several different ATPase activities. One of these is located in the fine membranous tubules of the L-system and was suggested to be associated with sequestration of Ca^{++} by the tubules. The other is located near the triad and was suggested to be associated with release of Ca^{++} from the lateral cisternae. Thus, in Gauthier's scheme, Ca^{++} is bound by the small tubules of the longitudinal system and is then transported along these tubules to the lateral cisternae, where it is stored until released in response to a nerve stimulus.

Consequently, the current concepts of muscle contraction suggest that the nerve impulse spreads as a wave of depolarization through the sarcolemma on the surface of the muscle cell. This wave of depolarization is conducted quickly throughout the muscle fiber by the T-tubules. At the junction of the T-tubules with the lateral cisternae of the L-system (the triads), the impulse is transmitted to the L-system as some kind of signal whose nature is presently unknown. This signal results in release of Ca^{++} from the membranous tubules of the longitudinal system. This Ca^{++} is then bound to

troponin on the thin filaments. The binding of Ca^{++} causes a conformational change in troponin which in turn uncovers a site on the actin filament. The myosin cross-bridge interacts with this site and contraction begins. When the signal to the lateral cisternae stops, the release of Ca^{++} stops, and Ca^{++} is rebound by the L-system. Troponin then covers all the myosin-binding sites on actin and contraction stops.

Post-mortem Muscle

Now that the current concepts of muscle action have been reviewed, the preceding concepts of muscle action will be used to help interpret and clarify some of the research on post-mortem muscle. A thorough knowledge of the thick and thin filament structure of muscle and the sliding filament theory of contraction is vitally important when attempting to understand the nature of post-mortem changes in muscle, particularly since Stromer and co-workers (Stromer and Goll, 1967a, 1967b; Stromer *et al.*, 1967) have shown that shortening of post-mortem muscle is accompanied by filament sliding in a manner structurally identical to that observed for *in vivo* contraction.

Although much of the early work on rigor mortis attributed carcass stiffening after death to lactic acid production and the resultant drop in pH, Hoet and Marks (1926) suggested that this was not the fundamental reason for carcass stiffening. They concluded that lactic acid would not cause rigor by itself but that it would affect the amount of shortening and tension produced. Thus, if an animal was exhausted during the death

struggle, very little lactic acid was produced and a "weak" rigor occurred. The idea that lactic acid had a fundamental causative role in stiffening during rigor mortis was disspelled by the Cambridge workers who shifted the emphasis in the study of rigor mortis from lactic acid to extensibility changes. Bate-Smith (1939) first reported that muscle changed from an elastic to an inelastic state as it passed through rigor mortis, and that this change in elasticity was paralleled by a decrease in pH. To facilitate further study of the extensibility changes during rigor, Bate-Smith developed a method for quantitatively measuring changes in muscle elasticity. This method consisted simply of periodically loading and unloading a muscle strip and recording on a kymograph the amount of stretch occurring in the strip. The method was later improved by Bate-Smith and Bendall (1949), who developed a procedure for automatically loading and unloading the muscle strip by electrical means. This instrument was further improved by Briskey et al., (1962) who added sealed chambers in which humidity, temperature and gaseous atmosphere could be controlled or changed. Because of the availability and widespread use of these instruments, rigor mortis has been almost universally defined in terms of extensibility; a muscle which has become inextensible is said to be in rigor mortis. Although these instruments were not specifically designed to measure shortening, the early workers noted that muscles often shortened as they passed into rigor mortis. However, the amount of shortening appeared quite variable in these early studies, and consequently, post-mortem shortening was not studied extensively until recently.

By use of the extensibility measurement, ante-mortem condition of

an animal was shown to have a marked effect on the time course of rigor mortis (Bate-Smith and Bendall, 1949). It was discovered that the type of rigor pattern (as indicated by changes in extensibility) depended on both the initial pH and the ultimate pH of the muscle. These pH values could be changed by altering either the nutritional or emotional state of the animal prior to slaughter. Severe stress just before slaughter resulted in a rapid loss of extensibility and a lower ultimate pH. When the animal is well-fed and undergoes little death struggle during slaughter, the pH decreases slowly from 6.9 - 7.2 at death to an ultimate pH of 5.5 - 6.0 after 48 - 72 hours post-mortem (Bodwell et al., 1965; Howard and Lawrie, 1956; Marsh, 1954). Under these conditions, loss of extensibility also occurs later post-mortem, and the muscle usually does not undergo severe shortening.

In 1943, Erdos (1943) showed that the decrease in elasticity of post-mortem muscle coincided with a post-mortem decrease in ATP concentration. This related the onset of rigor mortis, at least as defined in the extensibility sense, to ATP degradation. The role of energy-rich phosphate compounds in rigor mortis was also studied by Bate-Smith and Bendall (1947, 1949), who confirmed Erdos' finding that post-mortem muscle became inextensible only after it had lost its ATP. Moreover, Bate-Smith and Bendall (1949) were able to relate the rate of post-mortem ATP loss to the previously observed differences in rate of rigor development in stressed and unstressed animals. It was also shown that after death, when the supply of oxygenated blood to the muscle is interrupted, the muscle attempts to maintain its energy supply by anaerobic glycolysis. This anaerobic break-

down of glycogen to lactic acid is responsible for most of the pH decrease observed in post-mortem muscle and also serves to keep the ATP levels near those existing in the muscle at death. Eventually, however, glycogen reserves in the muscle are exhausted, and creatine phosphate (CP) must be used to maintain the muscle's ATP level. When CP reserves are exhausted, ATP concentration drops very quickly to levels of 0.1mM or less. In confirmation of this idea of post-mortem chemical changes in muscle, Bendall (1951) was able to show that ATP concentration remained near the at-death level until CP concentration had decreased to less than 30% of its at-death level. At this point, in situ enzyme systems apparently cannot efficiently rephosphorylate ADP from CP, and the ATP level falls rapidly to less than 20% of its initial concentration and then decreases slowly. Inextensibility and shortening occur during the rapid fall in ATP concentration, and Bendall (1951) was the first to suggest that post-mortem shortening of muscle may be a slow, irreversible contraction. In this scheme, loss of ATP, and consequently loss of extensibility, does not occur until after muscle glycogen and CP reserves have been depleted. Muscles of well-nourished, rested animals obviously contain greater amounts of glycogen than muscles of animals that have been severely stressed prior to slaughter. Thus, rigor mortis (in the extensibility sense) occurs sooner after death and at higher pH values in stressed animals than in well-fed rested animals. If an animal is severely stressed immediately prior to slaughter and slaughtered while still in the stressed condition, the blood stream is not able to remove lactic acid from the muscle before death. In such animals, initial pH is low and drops quickly to a low ultimate pH value soon

after death; rigor mortis occurs quickly.

These early concepts on the role of ATP in rigor mortis have remained virtually unchanged to the present time, although subsequent work has revealed some subtleties not noticed by the earlier investigators. It was shown by Straub and Feuer (1950) and by Laki (1957) that actin contains stoichiometric amounts of ATP which is dephosphorylated to ADP when actin is in the polymerized or fibrous form. Therefore, actin in living muscle contains substantial amounts of ADP, and indeed, Seraydarian et al., (1962) have shown that 70% of the ADP in muscle is in the bound form. Presumably all of this ADP is bound to actin. The importance of ADP in rigor mortis was first noticed by Kushmerick and Davies (1968), who discovered that inextensibility of post-mortem muscle required the absence of both ATP and ADP. This finding has subsequently been confirmed by Schmidt and Briskey (1968). It presently is not clear whether the absence of ADP means that the bound ADP of the actin filament must also be removed or whether degradation of sarcoplasmic ADP is sufficient.

Strohman (1959) first showed that the bound ADP of F-actin was not available for phosphorylation by the creatine kinase-creatine phosphate system. However, myofibrils contain actin in the fibrous or polymerized form, and Yagi and Noda (1960) found that myofibrillar ADP was phosphorylated by creatine kinase-creatine phosphate system, and that the phosphorylated nucleotide was then quickly hydrolyzed to ADP and inorganic phosphate. Later results by Moos (1964) suggested that deoxycholate, which had been used in Yagi and Noda's (1960) experiments, caused the release of some ADP from myofibrils, and that it was the free ADP which was being phosphorylated

by the creatine kinase-creatine phosphate system. Thus, the role and changes of the actin-bound ADP in muscle contraction and rigor mortis remain unclear.

Although it was shown that loss of ATP is closely associated with loss of extensibility in post-mortem muscle, the role of ATP in rigor shortening remains unclear. Indeed, it was not until 1962 that Davies and co-workers (Cain and Davies, 1962) were able to show that ATP was the primary energy source for muscle contraction. This fundamental demonstration was made possible by the discovery that 1 fluoro-2,4-dinitrobenzene (FDNB) almost completely inhibited creatine kinase without affecting the ATPase activity or contractile properties of muscle. Thus, after a muscle had been reacted with FDNB to prevent creatine kinase from rephosphorylating ATP, it was possible to show that ADP concentration in this muscle decreased upon contraction, but that CP concentration did not change. By incubating isolated frog muscles in 0.38mM FDNB for 90 minutes at 0^{o}, Nauss and Davies (1966) were able to show that ATP is broken down rapidly during the shortening phase of rigor, but that tension maintenance after rigor shortening did not require ATP. Therefore, post-mortem loss of ATP is a central factor in both the inextensibility and the shortening aspects of rigor.

During the 1950's, several investigators studied the characteristics of rigor development in several species other than the rabbit. Lawrie (1953) showed that ATP concentration at the time that post-mortem horse muscle becomes inextensible is about 30% of at-death ATP concentrations whereas ATP concentration in post-mortem rabbit muscle at the time it be-

comes inextensible is at considerably higher levels. Lawrie also found that the amount of glycogen and creatine phosphate available for ATP resynthesis appears directly related to the activity of the individual muscle and indirectly related to its capacity for aerobic glycolysis. Marsh (1952b) observed that whale muscle, during the onset of rigor mortis, changes from a dry state to an extremely wet state where the surface of the muscle was moist. This change in water retention was apparently related to the dephosphorylation of ATP.

In another later study, Marsh (1954) found that beef muscle showed a much greater change in modulus of elasticity than did rabbit. Otherwise, in the temperature range of 17-37°, beef muscle exhibited the same post-mortem changes described earlier for rabbit muscle. Marsh and Thompson (1958) studied lamb muscle and found that the rigor pattern in lamb was very similar to that in beef. Studies on rigor mortis in poultry by de Fremery and Pool (1960) showed that the same general changes occurred in poultry muscle as in beef or rabbit muscle with the exception that the entire rigor process occurred much more quickly in poultry.

Both the rate and extent of rigor development is markedly affected by storage temperature. Bate-Smith and Bendall (1949) first showed that rabbit muscle at 37° shortened 2.5 times more than muscle stored at 17°. Marsh (1954) measured shortening of bovine muscle strips at temperatures from 7° to 37° and found that the amount of work done in terms of shortening against a load increased progressively as the temperature was increased from 7° to 37°.

In 1963, a significant discovery concerning the effect of temperature on rigor development was made by Locker and Hagyard (1963). These in-

vestigators found that post mortem bovine muscle shortened maximally at 2^{o}, exhibited minimal shortening in the range 14-19^{o}, and then exhibited an increasing amount of shortening in the range 19-37^{o}. This was in marked contrast to post-mortem rabbit muscle, which shortened minimally at 2^{o}, and exhibited an increasing amount of shortening as the temperature increased from 2^{o} to 37^{o}. The shortening in bovine muscle at 2^{o}, termed "cold shortening", appeared to be different from normal rigor shortening in several respects: 1) it occurred while ATP concentrations were 1-2mM or more, and 2) it was reversible for some time after death. This reversibility was demonstrated by moving the muscle from a warm environment to a cold environment and then returning it to a warm temperature again. During these changes, the muscle would first shorten and then lengthen. This cycle could be repeated as long as ATP remained. As post-mortem time increased, however, this effect was diminished, which might indicate that ATP levels play an important role in this type of shortening.

Shortly after Locker and Hagyard discovered the cold shortening effect in post-mortem bovine muscle, Goll et al., (1964) reported that post-mortem changes in tenderness of bovine muscle were very dependent on whether the muscle had been excised immediately after death or left attached to the skeleton. Thus, in muscle excised from the skeleton immediately after death, tenderness decreased during the first 6 to 48 hours post-mortem and then increased slowly. On the other hand, muscles left attached to the skeleton were least tender immediately after death and gradually increased in tenderness with increasing post-mortem time. Even after 13 days, the muscle left attached to the skeleton was significantly more tender than

the muscle which had been excised immediately post-mortem. Although the excised muscle had obviously shortened considerably after 6-48 hours post-mortem, the differences in tenderness between the excised and attached muscles could not be related simply to extent of shortening alone. All tenderness tests were done on cooked muscle, and cooking of at-death muscle causes severe and extensive shortening. Consequently, the cooked samples of at-death muscle which were used for tenderness tests were far more extensively shortened than the cooked samples of the 6-48 hour excised muscle. Goll's results may be explained by Marsh and Leet's (1966) finding that tenderness of bovine muscle is not significantly affected if post-mortem muscle shortens only slightly down to 80% of its rest length. Tenderness is markedly decreased if it shortens to 60-80% of its rest length, and is actually increased if it shortens severely down to 40-60% of its rest length. Evidently, some shortening causes an increased density of thick and thin filaments per unit of cross-sectional area in the muscle and results in decreased tenderness. Shortening to less than 40% of rest length apparently causes disruption of Z-lines and other structural elements which hold the sarcomeres in proper juxtaposition; this results in increased tenderness.

Marsh and Leet's (1966) findings also offer a simple explanation for the results of Herring et al. (1965). These investigators found that muscle excised pre-rigor and stored at either 1^{o} or 5^{o} had sarcomere lengths 50% shorter than muscle excised post-rigor, and furthermore that muscle excised post-rigor was always more tender than muscle excised pre-

rigor. Moreover, psoas muscle, which is a very tender muscle, had considerably longer sarcomere lengths than semitendinosus, which is nearly always less tender than the psoas. If the muscle was restrained to prevent post-mortem shortening, a wide variation in sarcomere lengths was found, even though the muscle had been given a uniform treatment. These results of Herring et al. (1965) may be easily explained by Marsh and Leet's (1966) finding that, in muscle held at fixed length, different rates of cooling in different parts of the muscle cause the more rapidly cooled areas to shorten considerably, whereas other areas in the same muscle lengthen a compensatory amount. Fibers on the exterior of the restrained muscles in Herring's study probably underwent extensive shortening since they were cooled rapidly. Fibers on the interior of the muscle would not shorten to the same extent since they were not cooled as quickly post-mortem. This would cause the large variation in sarcomere lengths observed in these muscles by Herring et al. (1965).

Because of reports indicating that post-mortem tenderness changes were dependent on muscle lengths, Jungk et al. (1967) initiated some experiments in which muscle strips were held at constant lengths as they passed into rigor mortis. These experiments were made possible by development of an instrument called an isometer which measured isometric tension development of a post-mortem muscle strip during the onset of rigor mortis. With this instrument, it was shown that tension development in muscle strips held isometrically occurred at approximately the same time post-mortem as shortening in excised muscles. Furthermore, isometric tension

developed by post-mortem bovine muscle strips incubated in a humid chamber was maximal at 2°, minimal at 16°, and intermediate at 37° (Busch et al. 1967). Tension development in bovine muscle at 2° occurred much sooner post-mortem than it did at higher temperatures. On the other hand, isometric tension development of rabbit muscle was minimal at 2°, was intermediate at 16°, and was maximal at 37° (Busch, 1966). Thus, the amount of isometric tension development in post-mortem muscle appeared to approximate the extent of post-mortem shortening.

In their study on isometric tension development of post-mortem bovine muscle, Busch et al. (1967) measured ATP concentration, pH, and tenderness on portions of muscle samples held under conditions identical to those experienced by muscle strips on which isometric tension measurements were made. This made it possible to directly relate ATP concentration, pH, tenderness, and isometric tension development at several different post-mortem storage temperatures. Several fundamental differences were noticed between tension development at 2° and tension development at 37°: 1) tension development at 2° occurred in the presence of 5-6mM ATP, but tension development at 37° occurred in the presence of 1mM ATP or less, and 2) tension development at 37° began at pH values below 6.0 whereas tension development at 2° began at pH values above 6.0. Since it is difficult to see how a sliding of filaments could occur at ATP levels of 1mM or less, it was suggested that filament sliding according to Huxley and Hanson's (1954) model may occur only during post-mortem shortening at 2°, and that a different type of shortening or shrinkage occurs in post-mortem bovine muscle at 37°. However, electron microscopy studies of post-mortem bovine muscle

strips subsequently demonstrated that a sliding of filaments occurs during post-mortem shortening at either 2° or 37° (Stromer et al. 1967).

The electron microscopic studies of post-mortem muscle also showed that two important structural changes occur in post-mortem muscle: 1) a decrease in sarcomere length, and 2) a loss of Z-line integrity. The extent of these two changes depends on species and temperature. Thus, after 24 hours at 2°, both bovine myofibrils (Stromer and Goll, 1967a) and sectioned bovine muscle (Stromer et al., 1967) were supercontracted with thick filaments either passing through the Z-line or bent back upon themselves in the region of the Z-line. Sarcomere lengths of 2°-24 hour bovine muscle were about 1.2 μ (Stromer et al., 1967). Electron micrographs of post-mortem bovine muscle stored at 16° showed that this muscle had shortened only slightly. The 16°-24 hour bovine muscle had sarcomeres approximately 2.0 μ in length compared to sarcomere lengths of 2.7 μ for at-death bovine muscle. Bovine muscle after 24 hours at 37° had also shortened extensively to an average sarcomere length of 1.5 μ. The 37°-24 hour bovine muscle appeared severely disrupted, and the Z-line was difficult to see.

In an extensive examination of the ultrastructure of post-mortem muscle, Henderson (1968) extended Stromer's (Stromer and Goll, 1967b; Stromer et al., 1967) findings on bovine muscle to post-mortem porcine and rabbit muscle. In general, these electron microscopic studies confirmed the results of previous experiments on isometric tension development of porcine (Galloway and Goll, 1967) and rabbit muscle (Busch, 1966). Thus, sarcomere shortening was greatest at 37° in both rabbit and porcine muscle. Post-mortem shortening in rabbit muscle was least at 2° and increased

very slightly up to 25°, whereas post-mortem shortening of porcine muscle was minimal in the range 16-25°, and increased slightly between 16° and 2°. Sarcomere lengths observed in Henderson's microscopic study were not closely correlated to amount of isometric tension development since post-mortem rabbit muscle at 2° shortened considerably from sarcomere lengths of 2.5 μ at death to 1.6 μ after 24 hours. However, rabbit muscle strips at 2° developed almost no isometric tension. This discrepancy was resolved by the suggestion that a very small amount of tension development in unrestrained post-mortem muscle may cause shortening down to sarcomere lengths of 1.6 μ. However, shortening of unrestrained muscle past this point may require considerably greater forces due to increased resistance within the sliding filament mechanism itself. This increased resistance presumably arises from "frictional" forces due to extensive overlap of the interdigitating filaments, and to the force required to push thick filaments through the Z-line. Thus, shortening to sarcomere lengths less than 1.6 μ may require substantial tension development, even in unrestrained muscles.

Henderson's microscopic study also clearly showed thin filament overlap in the center of the A-band of post-mortem muscle which had shortened at 37°. This substantiates Stromer's (Stromer et al., 1967) finding that shortening at 37° occurs via a sliding of filaments, in contrast to the suggestion of Busch et al. (1967).

In addition to the findings on extent of sarcomere shortening in post-mortem muscle, Henderson's (1968) electron micrographs clearly indicated that Z-line degradation in post-mortem muscle occurred much sooner

post mortem and was much more extensive at temperatures of 37° than at 2°. Stromer et al. (1967) had earlier shown that Z-lines in bovine muscle lost their fibrillar structure and become amorphous during post-mortem storage at 2°. Henderson (1968) found that Z-lines of porcine or rabbit muscle were more labile to post-mortem storage than bovine muscle Z-lines, and that in some cases, rabbit or porcine Z-lines were absent altogether after 24 hours at 25° or 37°. Thus, both species and temperature have important effects on shortening and Z-line degradation in post-mortem muscle.

Some studies on solubility of myofibrillar proteins from post-mortem muscle have also provided evidence for post-mortem degradation of the Z-line, although this oftentimes was not realized at the time the studies were done. It has now been shown (Banga and Szent Gyorgyi, 1941; Haga et al., 1966; Mihalyi and Rowe, 1966) that high ionic strength salt solutions first extract myosin from minced muscle and that actin extraction must be preceded by a rupture of the bonds which hold actin to the Z-line. Thus, rate of actomyosin extraction, as distinguished from myosin extraction, constitutes an estimate of how rapidly the I-Z bonds are ruptured. This principle is clearly delineated here since many investigators have used extractability of actomyosin from post-mortem muscle as a measure of the state of the actin-myosin interaction, when in fact, there does not appear to be any reason to expect that the actin-myosin interaction would have any effect on myofibrillar protein solubility at all. Recently, Davey and Gilbert (1968) and Chaudhry et al. (1969) have found that

extractability of actomyosin increases with increasing time post-mortem. This is particularly noticeable at higher temperatures where Henderson (1968) observed that Z-line degradation was most severe. By differential extraction techniques, Davey and Gilbert (1968) were able to obtain evidence that solubility of bovine myosin remained constant during 21 days of post-mortem aging at 2°, but that solubility of actin and a third unidentified protein increased during this period. Penny (1968) also found that the amount of actin and tropomyosin extracted from rabbit myofibrils by a 1M KCl solution at pH 6.2 increased with increasing post-mortem time at either 2° or 15-18°, whereas the extractability of myosin alone remained unchanged during this period. These extraction studies, therefore, suggest that the bonds between the I- and Z-filaments are weakened in post-mortem muscle and result in more complete extraction of actin.

Temperature has been shown to have other unusual effects on post-mortem muscle. It has generally been assumed that post-mortem glycolysis occurs more rapidly at higher temperatures, and consequently that at higher post-mortem storage temperature, ATP, glycogen, and CP levels would decrease sooner post-mortem, and pH would decrease more rapidly than at lower storage temperature. Therefore, the onset of rigor would occur more quickly at temperatures between 25 and 37° than at temperatures between 0 and 20°. Indeed, Bate-Smith and Bendall (1949) found this to be true for rabbit muscle in the range of 17-37°. Furthermore, Disney et al. (1967) found that rapid cooling, accomplished by placing bovine muscles in ice immediately post-mortem, retarded post-mortem glycolysis and ATP degradation in these muscles. This rapid cooling of post-mortem bovine tissue

also resulted in less protein denaturation and increased water holding capacity of the tissue.

In contrast to these ideas, Cassens and Newbold (1966) found that during the first several hours post-mortem, the rate of decrease of pH, CP, and acid-labile phosphate in bovine sternomandibularis muscle at 1° was the same as that at 15°. However, pH, CP, and acid-labile phosphate levels all decreased much more rapidly at 37° than at 15°. Subsequently, Cassens and Newbold (1967a) found that the pH of bovine sternomandibularis muscle actually decreased more rapidly at 1° than at 5°. Measurements of alkali-labile phosphate indicated that greater amounts of hexose-6-phosphates accumulate in post-mortem muscle at 1° than at 15°, and Cassens and Newbold (1966) suggested that the breakdown of hexose-6-phosphate was the rate-limiting step in post-mortem glycolysis. Evidently, breakdown of hexose-6-phosphate occurs unusually slowly at 1°, and this results in less ATP production by post-mortem glycolysis and a relatively fast rate of ATP breakdown at 1°. de Fremery and Pool (1960) found that ATP in the chicken pectoralis major muscle decreased more rapidly at 0° than at temperatures in the range of 10-30°. Also, Busch et al. (1967), found that rate of ATP breakdown in bovine psoas or semitendinosus muscles was similar at 2° and 16°, but was much faster at 37° than at 16°. Furthermore, Cassens and Newbold (1967b) found that although the delay phase of rigor in bovine sternomandibularis muscle was longer with lower temperatures in the 15-37° range, it was shorter with lower temperature in the 1-15° range. Moreover, the onset phase of rigor was longer with lower temperature in the 5-37° range, but was shorter with lower temperature in the

1-5° range.

It is interesting to note that these unusual rates of post-mortem glycolysis in chicken and bovine muscle between 1 and 10° are paralleled by the unusual cold shortening effect in muscle from these species (Locker 1960; Lowe, 1948). Rabbit muscle, on the other hand, does not exhibit a cold shortening effect (Busch, 1966). Bendall (1960) has shown that ATP levels in post-mortem rabbit muscle decrease more rapidly as the temperature is raised from 0-37°, in agreement with the expected effect of temperature on post-mortem glycolysis. In spite of this evidence, it is difficult at the present time, to accurately delineate any causative relation between cold shortening and the unusual temperature effects on post-mortem glycolysis in bovine and chicken muscle.

Although the isometric tension technique was very valuable in elucidation of tension patterns in post-mortem muscle, this was not the most significant contribution of this technique. Soon after the initial use of isometric tension in characterizing post-mortem isometric tension patterns, it was discovered that if post-mortem muscle strips remained attached to the instrument for 24-48 hours, a gradual loss in ability of the strips to maintain their isometric tension development could be detected (Jungk et al., 1967; Busch et al., 1967). This loss in ability to maintain isometric tension was noticed first in bovine muscle at 2° where tension development was greater and where a low temperature delayed drying of the strip (Busch et al., 1967). Soon afterward, however, it was found that some loss in ability to maintain isometric tension also occurred at higher post-mortem temperatures (Busch et al., 1967; Goll, 1968).

This loss in ability to maintain isometric tension is the first measurable physical property that can be related to the widely discussed "resolution of rigor". Meat scientists had consistently noticed that excised bovine muscles first decreased in tenderness, and then, after 6-48 hours post-mortem, gradually increased in tenderness (Paul et al., 1948; Goll et al., 1964). The gradual increase in tenderness was termed a "resolution of rigor", since it presumably originated from loss of carcass stiffness. However, when the Cambridge group developed the extensibility measure of rigor mortis (Bate-Smith, 1939), it was shown that once post-mortem muscle had become inextensible, it did not regain its extensibility even if it was stored under aseptic conditions for many days. Thus, when rigor is defined solely in terms of extensibility, there is no resolution of rigor mortis, and the cause of the post-mortem tenderization seen so clearly in post-mortem bovine muscle became shrouded in controversy.

Recently, it has been suggested that rigor mortis should be defined in terms of post-mortem tension development, since it seems likely that tension development by two muscles on opposing sides of the same bone would cause carcass stiffness (Briskey, 1964); Goll, 1968; Goll and Robson, 1967). Furthermore, the words "rigor mortis" literally mean "stiffness of death", and there is no clear reason why loss of extensibility should by itself cause muscle stiffness. If rigor mortis is defined in terms of tension development, then it is clear that loss of the ability to maintain isometric tension corresponds to a "resolution of rigor".

The cause of the loss in ability to maintain isometric tension remains unclear, although there has been much new information obtained on this

question. Recently, Goll (1968) has proposed that two alterations in molecular architecture of the myofibril may be responsible for the resolution of rigor mortis (when rigor is defined in terms of tension). These two changes are: 1) loss of Z-line structure and weakening and eventual rupture of the bonds between the I and Z-filaments, and 2) a weakening of the actin-myosin interaction, this weakening possibly resulting in some partial increase in post-mortem muscle extensibility.

There is considerable evidence supporting Goll's (1968) suggestion that the Z-line loses its integrity in post-mortem muscle. The electron microscopic studies of Stromer et al. (1967) and Henderson (1968) which show loss of Z-line structure in post-mortem bovine, porcine, and rabbit muscle, have already been mentioned. Furthermore, Takahashi et al. (1967), Stromer and Goll (1967a), and Henderson (1968) have reported that myofibrils prepared from muscle after 2-3 days of post-mortem storage appear highly fragmented, with many of these being only 1-3 sarcomeres in length, whereas myofibrils prepared from at-death muscle contain 8-10 or more sarcomeres. This fragmentation of post-mortem myofibrils occurs primarily at the Z-line, which suggests that post-mortem myofibrils are weakened at this point so that they break or rupture during the homogenization procedure used in myofibril preparation. Recently, Davey and Gilbert (1969) have observed that Z-line in post-mortem bovine muscle may be removed from the myofibril altogether and that adjacent myofibrils appear to lose their affinity for each other.

Although it is clear from these studies that extensive degradation

of Z-lines occurs in post-mortem muscle, the cause for this degradation remains an enigma. The Z-line has been shown to be very susceptible to removal by proteolytic enzymes such as trypsin (Stromer et al., 1967), so it might be expected that proteolysis by cathepsins would be a likely cause for Z-line degradation in post-mortem muscle. Fukazawa and Yasui (1967), however, have reported that addition of partially purified cathep-tic enzymes to chicken myofibrils does not cause any noticeable structural alterations in the Z-line. Moreover, Stromer et al. (1969) were able to selectively remove the Z-line from glycerinated rabbit psoas fibers by extraction with very low ionic strength solutions. Thus, it is possible that Z-line degradation results from prolonged exposures to nonphysiologi-cal temperatures and pH values. Regardless of its origin, it seems reason-able to suppose that loss of Z-line integrity and rupture of the I-Z bonds in post mortem muscle should result in increased tenderness and may also contribute to loss of the ability to maintain isometric tension.

The second alteration which Goll (1968) suggested as a primary con-tributor to the resolution of rigor mortis in muscle is a weakening of the actin-myosin interaction, which in turn leads to some increase in post-mortem muscle extensibility. Goll attempts to distinguish his proposed weakening of the actin-myosin interaction from earlier theories that sug-gested that the actin-myosin interaction was completely dissociated during the resolution of rigor mortis (Partman, 1963; Weinberg and Rose, 1960; Wierbicki et al., 1954). However, Wierbicki et al. (1956) could find no evidence to support their earlier proposal that the actin-myosin

interaction was completely dissociated during the resolution of rigor mortis. The fact that post-mortem muscle never fully regains its original extensibility argues conclusively against a complete dissociation of the actin-myosin complex during the resolution of rigor. Therefore, Goll suggests that the actin-myosin interaction is weakened in post-mortem muscle, so that traces of residual ATP, ADP, or other agents *in situ* could cause some "slippage" or partial dissociation at those points where myosin cross-bridges interact with the actin filament. Direct evidence favoring Goll's proposal was obtained by Fujimaki, Arakawa, and co-workers (Fujimaki *et al.* 1956a, b) who found that, in the presence of 1mM Mg^{++} and 600mM KCl, myosin B prepared from rabbit muscle after 7 days post-mortem was dissociated to actin and myosin by 0.1mM ATP, whereas 0.6mM ATP was required to cause this same dissociation in myosin B prepared from at-death muscle. Recently, Goll and Robson (1967) and Robson *et al.* (1967) found that the Mg^{++} - or Ca^{++}-modified ATPase activities of myofibrils or myosin B prepared from bovine muscle after 24 hours post-mortem at 2^{o} or 16^{o} are 20-50% higher than the corresponding activities of myofibrils or myosin B prepared from at-death bovine muscle. A possible cause for this increased ATPase activity was presented when it was discovered that very brief tryptic digestion of at-death myofibrils also causes a 50-80% increase in the Mg^{++}- or Ca^{++}-modified ATPase activities of myofibrils. Moreover, brief tryptic digestion of contracted myofibrils in the absence of ATP causes lengthening of the shortened myofibrils from sarcomere lengths of 1.0 μ to lengths of 1.7 μ (Goll, 1968). This trypsin-induced

lengthening of contracted myofibrils resembles the lengthening observed during post-mortem aging of bovine muscle (Gothard et al., 1966; Stromer et al., 1967) or chicken muscle (Takahashi et al., 1967) because both occur in the absence of measurable amounts of ATP. Consequently, these results suggest that a very limited and specific catheptic proteolysis of actin, myosin, and/or one of the regulatory proteins may cause weakening of the actin-myosin interaction in post-mortem muscle.

Although cathepsins have long been implicated as a causative agent in post-mortem tenderization, it has been very difficult to obtain unequivocal evidence that cathepsins have any effect on the myofibrillar proteins. In fact, all of the available evidence suggests that if cathepsins are active in proteolysis of post-mortem muscle, their activity is limited to an effect on the sarcoplasmic fraction, and they do not hydrolyze myofibrillar proteins (Bodwell and Pearson, 1964; Davey and Gilbert, 1966; Parrish et al., 1969; Sharp, 1963; Suzuki et al., 1967). There is ample evidence that cathepsins are present in post-mortem muscle (Iodice et al., 1966; Martins and Whitaker, 1968; Parrish and Bailey, 1966; Suzuki and Fujimaki, 1968), and several studies have described their purification and activity against synthetic substrates. Martins and Whitaker (1968) have demonstrated that a highly purified cathepsin isolated from chicken muscle and purified 580-fold had no detectable activity on a natural actomyosin substrate, even though several different kinds of sensitive tests were used in an effort to detect such proteolysis. Although Locker (1960) found increases in N-terminal amino groups in bovine

muscle stored for 14 days at 2° or 3 days at 21°, these increases were very small, and other results indicated no evidence of proteolysis. Thus, Locker concluded that the tenderization which occurred with aging was due to some mechanism other than proteolysis. Furthermore, both Davey and Gilbert (1966) and Parrish et al. (1969) have pointed out that the increase in non-protein nitrogen or free amino acids, which is frequently observed in post-mortem muscle, occurs only after 3-5 days of storage at 2°. On the other hand, the post-mortem increase in tenderness in these muscles usually occurs between 6 and 72 hours post-mortem, before any increase in free amino acids is detectable. Thus, even though some proteolysis is often detectable in post-mortem muscle, this proteolysis presently cannot be related to post-mortem tenderization.

The problem of proteolysis of myofibrillar proteins has been discussed by Parrish et al. (1969). As these investigators point out, there are three myofibrillar sites that, based on their known vulnerability to trypsin, might be expected to be affected by cathepsins. The first of these is myosin itself, which is quickly cleaved into two macromolecular fragments, light and heavy meromyosin, by either trypsin or chymotrypsin. Since both Martins and Whitaker (1968) and Bodwell and Pearson (1964) have shown that neither actomyosin nor myosin is affected by purified cathepsins, it is unlikely that any proteolysis of myosin occurs in post-mortem muscle. This conclusion is substantiated by the finding that ATPase activity of post-mortem muscle does not undergo large changes (Goll and Robson, 1967; Penny, 1968; Robson et al., 1967) as would be expected if myosin had been

proteolytically cleaved.

The second proteolytically-labile site is the tropomyosin-troponin complex. This complex has been shown to be rapidly degraded to small peptides by trypsin (Laki, 1957; Ebashi and Kodama, 1966). However, biochemical evidence on Ca^{++} sensitivity of the ATPase activity of post-mortem myofibrils has shown that the tropomyosin-troponin complex is not proteolytically destroyed, even after 13 days post-mortem at 16^{o} (Galloway and Goll, 1967; Goll and Robson, 1967). This conclusion is substantiated by Davey and Gilbert's (1966) suggestion that tropomyosin remains difficult to extract, even after 21 days post-mortem at 2^{o}.

The third possible site for post-mortem proteolysis of myofibrils is at or near the Z-line. The susceptibility of this region to trypsin has already been discussed, and all experiments attempting to demonstrate that cathepsins will remove the Z-line have failed (Fukazawa and Yasui, 1967). Consequently, all evidence available thus far suggests that there is no extensive catheptic proteolysis of the myofibril in post-mortem muscle, and it must be concluded that if there is any post-mortem proteolytic effect at all on the myofibrillar proteins, it must be an extremely limited and unusual effect, perhaps similar to that proposed by Goll (1968) as being responsible for weakening of the actin-myosin interaction.

A very limited amount of work on changes occurring in the sarcoplasmic reticulum upon aging has recently been reported. Baird and Perry (1960) and Ebashi and Lipman (1962) noted that sarcoplasmic reticular vesicles isolated by differential centrifugation lost their ability to

inhibit contraction after several days storage at 0°. The first attempt to relate the known lability of sarcoplasmic reticular vesicles to possible changes in the sarcoplasmic reticulum in post-mortem muscle was reported by Greaser et al. (1967). These investigators found that the heavy sarcoplasmic fraction, which was separated by centrifugation between 8,000 to 30,000xg, lost 40% of its Ca^{++}-sequestering ability within the first three hours post-mortem, with the largest decrease occurring between the second and third hours post-mortem. This large initial decrease is interesting since it is during this period that post-mortem muscle pH drops considerably, but the carcass temperature has not yet dropped below 25°. It is also during this period that rigor shortening is initiated. Therefore, Greaser's finding suggests that Ca^{++} acts as the trigger for post-mortem shortening, since inactivation of the sarcoplasmic Ca^{++} pump would result in the release of Ca^{++}.

Nauss and Davies (1966) have obtained more direct evidence on this point by using FDNB-poisoned frog sartorius muscles. They showed that the onset of rigor shortening was accompanied by an increase in the rate of Ca^{++} efflux. In Greaser's study (Greaser et al., 1967) on sarcoplasmic reticular vesicles from post-mortem muscle, evidence was obtained that indicated that the loss in Ca^{++} accumulating ability of vesicles isolated from post-mortem muscle was due to inactivation of the Ca^{++} pump rather than disruption of the sarcoplasmic reticular membranes. Thus, electron micrographs indicated that the structure of vesicles prepared from post-mortem muscle was very similar to that of vesicles prepared from at-death muscle. Furthermore, protein contents of the different vesicular fractions

remained constant during post-mortem aging. Since ATP is rapidly degraded during the initial stages of rigor development, it seems possible that lack of ATP may be a factor causing inactivation of the Ca^{++} pump. However, Neelin and Ecobichon (1966) have suggested that some proteolytic destruction of the sarcoplasmic reticulum may occur during the first 24 hours post-mortem, and this may also be a causative agent in the loss of Ca^{++}-sequestering activity in post-mortem sarcoplasmic reticulum.

Two recent papers reported some unusual effects of added Ca^{++} chelators on the development of rigor mortis in muscle strips. Feinstein (1966) found that rigor in frog muscle, as defined in the tension development sense, could be inhibited by soaking muscles in a Ca^{++}-free Ringers solution containing various levels of EDTA or EGTA. The amount of inhibition was dependent upon the level of chelator used. Feinstein (1966) suggested that these chelators alter membrane structure considerably and are thereby able to penetrate the cell membrane and work intracellularly. Inhibition of rigor shortening was also noticed by Weiner and Pearson (1966) when a lethal ante-mortem injection of EDTA was given to a rabbit. Muscles from the treated animals maintained their extensibility and could be stretched to 40% of their rest length. The fibers were elastic since they returned to their original length after removal of the stretching force. ATP degradation and pH decline in muscles from the EDTA-treated animals were very similar to those in muscles from the control animals, indicating that removal of Ca^{++} by EDTA did not affect these parameters. These findings are difficult to interpret since the ability of EDTA or

EGTA to penetrate the muscle cell membrane remains unknown. It is clear, however, that Ca^{++} has marked effects on the development of rigor mortis although the nature and cause of these effects is not yet evident.

Red and White Muscle

Muscles with predominately red (Type I) fibers and muscles with predominately white (Type II) fibers have been shown to have very different properties. Sexton and Gersten (1967) have summarized the biochemical differences between these two types of fibers. Briefly, red fibers contain more myoglobin, greater mitochondrial activity, more sodium, less potassium, and exhibit a greater uptake of glucose and glycogen synthesis than do white fibers. Glycerinated red muscle fibers develop more isometric tension than white fibers. Cassens et al. (1967) found that red muscle contains three to four times more zinc than white muscle, and Beecher et al. (1965) reported that sarcomere lengths were generally longer in red than in white muscle. These differences are generally consistent with the idea that red fibers are capable of slow but sustained contractures. Such fibers must supply much of the ATP needed for their activity by their own glycolysis. Thus, they possess abundant glycolytic enzymes and an extensive oxygen supply, and have numerous mitochondria to use oxygen in aerobic glycolysis. White fibers, on the other hand, are fibers capable of very rapid but brief contractures. Such fibers depend largely on outside sources or their own anaerobic glycolysis for energy supply. Consequently, they are not richly supplied with oxygen-storage or oxygen-consuming systems.

Until 1964, it was generally thought that myofibrils of red and

white muscle were identical, and that the only differences between the two types of fibers were in the sarcoplasmic protein fraction. Then, in 1964, Seidel et al. (1964) indicated that the Mg^{++}-modified ATPase activity of myofibrils from white muscle was six-fold higher than the corresponding activity of myofibrils from red muscle. This effect was shown tc originate from a difference in the ATPase activity of myosin from red and white muscle. Subsequent work (Sreter et al., 1966) has shown that this difference is not due to contamination by inactive protein or to the presence of an ATPase inhibitor in the red muscle myosin. The enthalpy of activation of the Ca^{++}-modified ATPase activity of white myosin was much lower than that of red muscle. This suggests that white myosin must have a lower activation energy than myosin from red muscle. Barany et al. (1965) reported similar differences between the ATPase activity of red and white myosin. These investigators found that red muscle myosin had two or three times lower actin-activated and EDTA-activated ATPase and Ca^{++}-activated ATPase and ITPase activities than white muscle myosin. Studies on reactivity of sulfhydryl groups by Sreter et al. (1966) showed that more p-chloromercuribenzoate (PMB) was needed to cause ATPase activation in freshly prepared red muscle myosin than in freshly prepared white myosin; this difference was removed upon aging. This indicates that some sulfhydryl groups in red muscle myosin are initially more available to PMB than in white myosin, but that a structural change occurs with aging, and the readily accessible SH groups in red muscle myosin are lost.

There are also other indications that a very small structural difference may exist between red and white muscle myosin. The tryptic cleavage

of myosin into heavy and light meromyosins occurs at a slower rate in red muscle myosin than in white muscle myosin. Also, the ATPase activity of red muscle myosin is very labile under mild alkaline conditions (pH 9.0 for 10 minutes), but the ATPase activity of white muscle myosin is hardly affected by this treatment (Seidel, 1967). Inactivation of red muscle myosin is not accompanied by any detectable change in sedimentation rate, rate of trypsin digestion, or optical rotatory dispersion curves, which suggests that only very small changes in secondary or tertiary structures accompany the alkaline inactivation. The exact nature of these differences between myosin from red and white muscle remains unknown, but it is tempting to speculate that they may be due to differences in the small subunits recently discovered in the myosin molecule (Stracher and Dreizen, 1966). Frederiksen and Holtzer (1968) have shown that the small subunits in myosin are intimately involved in the ATPase and actin-binding properties of myosin, and Locker and Hagyard (1967) have found differences between the small subunit content of rabbit skeletal myosin (white) and rabbit cardiac myosin (red). However, the exact role of the small subunits and their relation to the differences between red and white muscle myosin remain to be elucidated.

Almost simultaneously with the discovery that myosin in red and white muscle differed, it was found that Ca^{++}-binding activity of grana isolated from the two types of muscles also differed (Sreter and Gergely, 1964). In the presence of oxalate, Ca^{++}-uptake of white muscle grana is ten times higher than that of red muscle grana. However, the initial Mg^{++}-modified ATPase activity of red grana in oxalate-free solutions was higher

than the corresponding activity for white grana. EDTA inhibited the Mg^{++}-modified ATPase activity of white grana but had no effect on the Mg^{++}-modified ATPase of red muscle grana. Aging of red grana for three weeks resulted in a decrease in Mg^{++}-modified ATPase activity, whereas the ATPase activity of white grana always increased during this treatment. Electron microscopy showed that red muscle grana consist principally of round vesicles with few narrow tubular elements, whereas white grana contain primarily narrow tubular elements.

In summary, although it is now appreciated that the contractile elements as well as the sarcoplasmic milieu differ in red and white muscle, the nature of these differences, particularly in the contractile elements, remains unknown. It presently appears that in white muscle, which is the more highly differentiated form, nature has modified the contractile elements themselves to enable them to more efficiently perform their task, i.e., to contract very rapidly.

METHODS AND MATERIALS

Source of Muscle Tissue

Rabbit psoas muscle was the primary source of muscle tissue for this study. Rabbits were anesthetized with sodium pentobarbital (90 mg) and d-tubocurarine chloride (1.5 mg) prior to exsanguination. Immediately after exsanguination, the psoas was removed and used for the various analyses.

Bovine semitendinosus, and porcine longissimus dorsi or semitendinosus muscles were obtained as soon as possible after exsanguination at the Iowa State University Meat Laboratory. The animals were not anesthetized, although bovine animals were stunned with a captive bolt pistol just before exsanguination. The excised muscles were taken to the Food Research Laboratory and used at once in the experiments described in the subsequent paragraphs. In the case of both bovine and porcine muscle tissue, the experiments were initiated about thirty minutes post-mortem, whereas with rabbit, the studies were started within ten minutes after death.

Tension Measurements

Tension development and release was measured with an E and M Physiograph (E and M Instrument Co., Inc., Houston, Texas) equipped with both isometric and isotonic myograph transducers. The environment of the muscle strips was controlled by immersing the strips in saline solutions near physiological ionic strength. An environmental control chamber (Labline Inc., Chicago, Illinois) was used to maintain a constant temperature for

each experiment.

For all tension measurements, muscle strips 0.1 to 0.3 cm^2 in cross section and approximately 7 cm long were removed from each muscle by carefully cutting parallel to the muscle fibers to minimize structural damage. The excised strips were then weighed, and one end was immediately attached to the transducer by surgical thread. The other end of the strip was securely fastened with surgical thread to a glass rod projecting from the side of an 800 ml beaker at a point approximately 15mm above the bottom. This arrangement allowed a magnetic stirrer to be used for mixing when various chemicals were added to the solution during the course of the experiment.

Initial studies on the effect of temperature on rigor mortis used a solution that consisted of 0.06 M phosphate buffer (pH 7.2), 0.05 M KCl, 0.005 M $MgCl_2$ and 0.001 M NaN_3. The NaN_3 was added to prevent bacterial growth, especially at the higher temperatures. For all subsequent studies, a standard solution, hereafter referred to as "solution B", was used. This solution consisted of 0.06 M Tris acetate, pH 7.1, 0.08 M KCl, 0.005 M $MgCl_2$ and 0.001 M NaN_3. The various additions made to this solution in different experiments will be given when describing the results of the experiments. In experiments where EDTA was added to the solution, the $MgCl_2$ was omitted from solution B.

Isometric tension studies were done by using isometric transducers supplied by the E and M Instrument Co. In order to keep each strip taut and to assist in maintaining uniformity of initial conditions, approximately 4 gm/cm^2 of tension was placed on each strip when it was first attached

to the isometric myograph. Five isometric transducers were used in each experiment, thus allowing up to five comparisons on any one animal. This facilitated studies on the effects of various treatments since animal variation was eliminated by this design. Considerable care was taken to prevent stretching of the muscle strips while they were being excised and mounted on the instrument.

Extensibility was measured with isotonic transducers. Since only a very limited study was done on this aspect, a 50-gm weight was manually loaded onto the fiber at 8-minute intervals. The weight was left on the fiber for two minutes each time. Strips with a cross sectional area of 0.5 cm^2 were used for this study.

The extent of shortening of muscle strips was measured by the same method used for isometric tension except that isotonic transducers were used. Ten grams of initial tension were placed on the strip by means of a counter weight. This was just enough tension to keep the fiber taut.

ATPase Determinations

The effect of storage at different pH values on myofibrillar ATPase activity was studied by using rabbit myofibrils. Ground at-death, rabbit muscle was homogenized in 0.25 M sucrose, 1mM EDTA, 0.05 M Tris, pH 7.6, and myofibrils prepared according to the method of Goll and Robson (1967). The preparation of the myofibrils was done at 2°, using precooled solutions. After preparation, the myofibrils were sedimented and washed two times with 0.05 M KCl, 0.05 M Tris acetate at one of six pH values: 5.5,

6.0, 6.5, 7.0, 7.5, 8.0. The myofibrils were then stored at 2° in the same salt solution and at the same pH value at which they had been washed. After specified storage periods of 24, 72 and 168 hrs, the myofibrils were sedimented, washed with five volumes of 0.15 M KCl, 0.10 M Tris acetate, pH 7.0, and then washed two more times with five volumes of 0.05 M KCl followed finally by resuspension in five volumes of 0.05 M KCl. Aliquots of this suspension were used for the ATPase assay according to the method of Goll and Robson (1967). Ionic environment for the individual experiments will be described in the "Results" section.

Phase Microscopy

Strips were removed from the isometric or isotonic transducers during pre-rigor, rigor, or post-rigor stages as judged by the state of development or release of isometric tension of the strips. Myofibrils were prepared from the strips using the method of Goll and Robson (1967), and were then examined with a Zeiss Photomicroscope equipped with phase optics. For all micrographs, a 100X planapochromatic objective was used with a green interference filter in the light path. Results were recorded on Adox KB-14 film.

Electron Microscopy

The ultrastructure of the muscle strips attached to the isometric or isotonic myographs was studied at various stages of tension development and under different ionic conditions. The strips were removed from the physiograph during either the pre-tension or maximum tension development

stages or after release of isometric tension and sub-divided along their longitudinal axis into strips 1-2 mm in diameter and 20-25 mm long. These strips were fixed according to the method of Stromer (Stromer et al., 1969) and then embedded in an Epon-Araldite mixture as described by Anderson and Ellis (1965). The blocks containing the embedded samples were polymerized at 60° for 54 hours, cured at room temperature for 2 - 3 days, and sectioned using a glass knife on an LKB Ultrotome III. All samples were oriented to give a longitudinal section of the muscle fiber. Sections approximately 60-75 mu in thickness were used. The sections were mounted on uncoated, 400 mesh grids, stained with 2% uranyl acetate for 20 minutes and washed 10 seconds in 75% methanol-water and 15 seconds in 50% methanol-water. This was followed by a 1 minute wash in distilled H_2O. The grids containing the sections were then thoroughly dried and post-stained with lead citrate for 11 minutes according to the procedure of Reynolds (1963). All electron micrographs were taken on an RCA EMU-4 instrument operated at 50 kv.

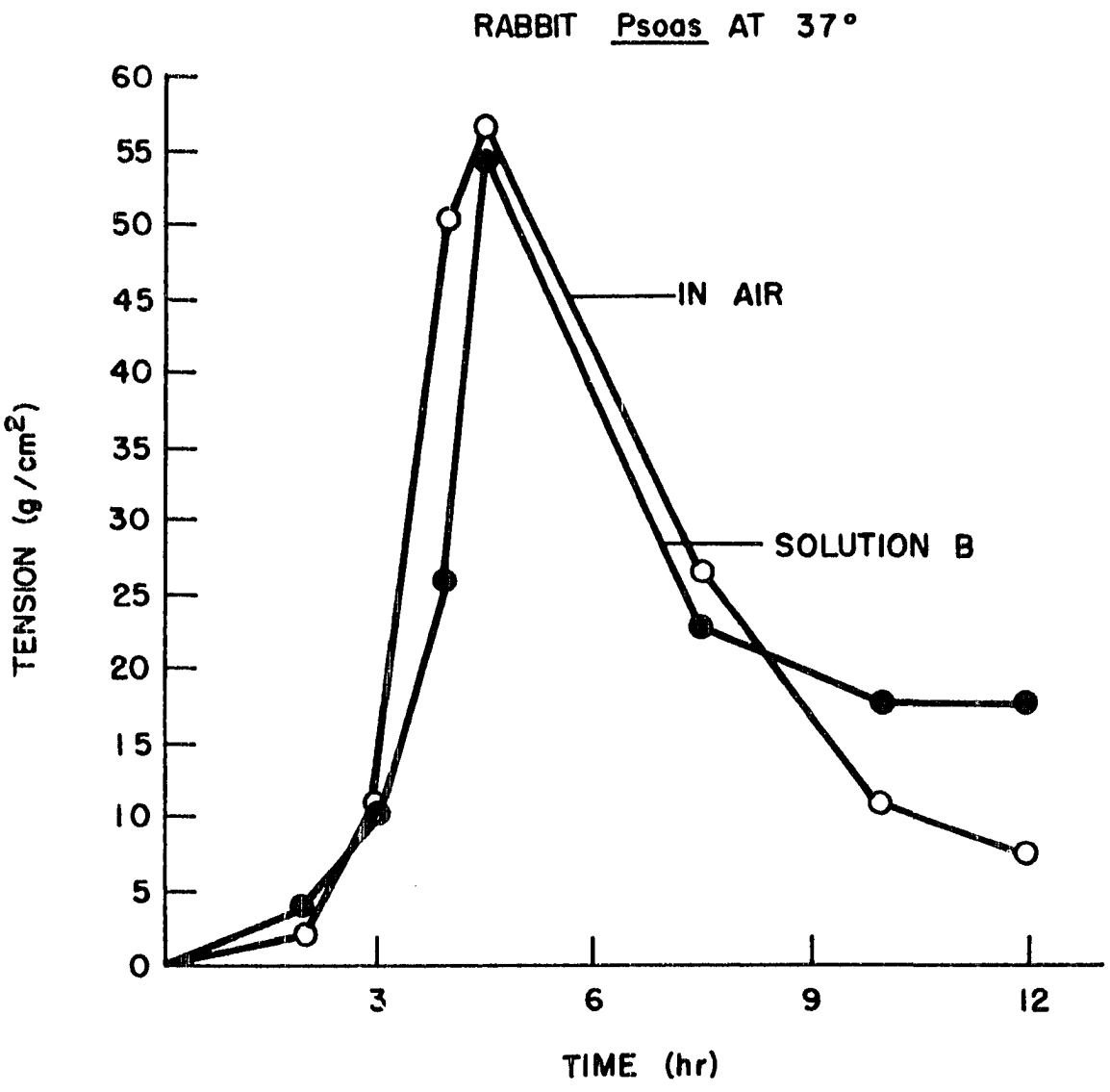
RESULTS

Several different types of studies were done in this investigation, and for clarity, the results of these different studies will be discussed separately under different headings. The order of presentation is approximately the same as the order in which the studies were done.

Effect of Temperature on Post-Mortem Isometric Tension Development

Earlier studies on isometric tension development in post-mortem muscle showed that both development and release of tension occurred at 2^{o}, but due to dehydration of the muscle strips, even when kept in a humid chamber, it was difficult to consistently demonstrate loss of ability to maintain isometric tension at higher temperatures. To circumvent this difficulty, this study tested the effect of complete immersion of muscle strips in a liquid bathing medium on the development and decline of post-mortem tension. Obviously, immersion of strips in a saline solution will obviate all dehydration problems. After experimenting with various saline solutions, it was found that post-mortem isometric tension patterns of strips immersed in 0.06M Tris, 0.08M KCl, 5mM $MgCl_2$, 1mM NaN_3, pH 7.1 (hereafter called "solution B") was very similar to the isometric tension pattern of strips kept in a humid chamber (Figure 1). For rabbit psoas muscles at 37^{o}, both strips in solution and strips in air began to develop isometric tension about 2 hours post-mortem and maximal tension occurred after 5 hours post-mortem. Both developed a maximum tension of nearly 55 g/cm^2, and the amount of isometric tension decreased rapidly

Figure 1. Comparison of post-mortem isometric tension development of muscle strips suspended in solution B or in humid air. Muscle strips were taken from rabbit psoas. Each point is an average of measurements made on four strips. Temperature of solution was 37°.


RABBIT Psoas AT 37°
TENSION (g/cm²)
60
55
50
45
40
35
30
25
20
15
10
5
0
3
6
9
12
TIME (hr)
IN AIR
SOLUTION B

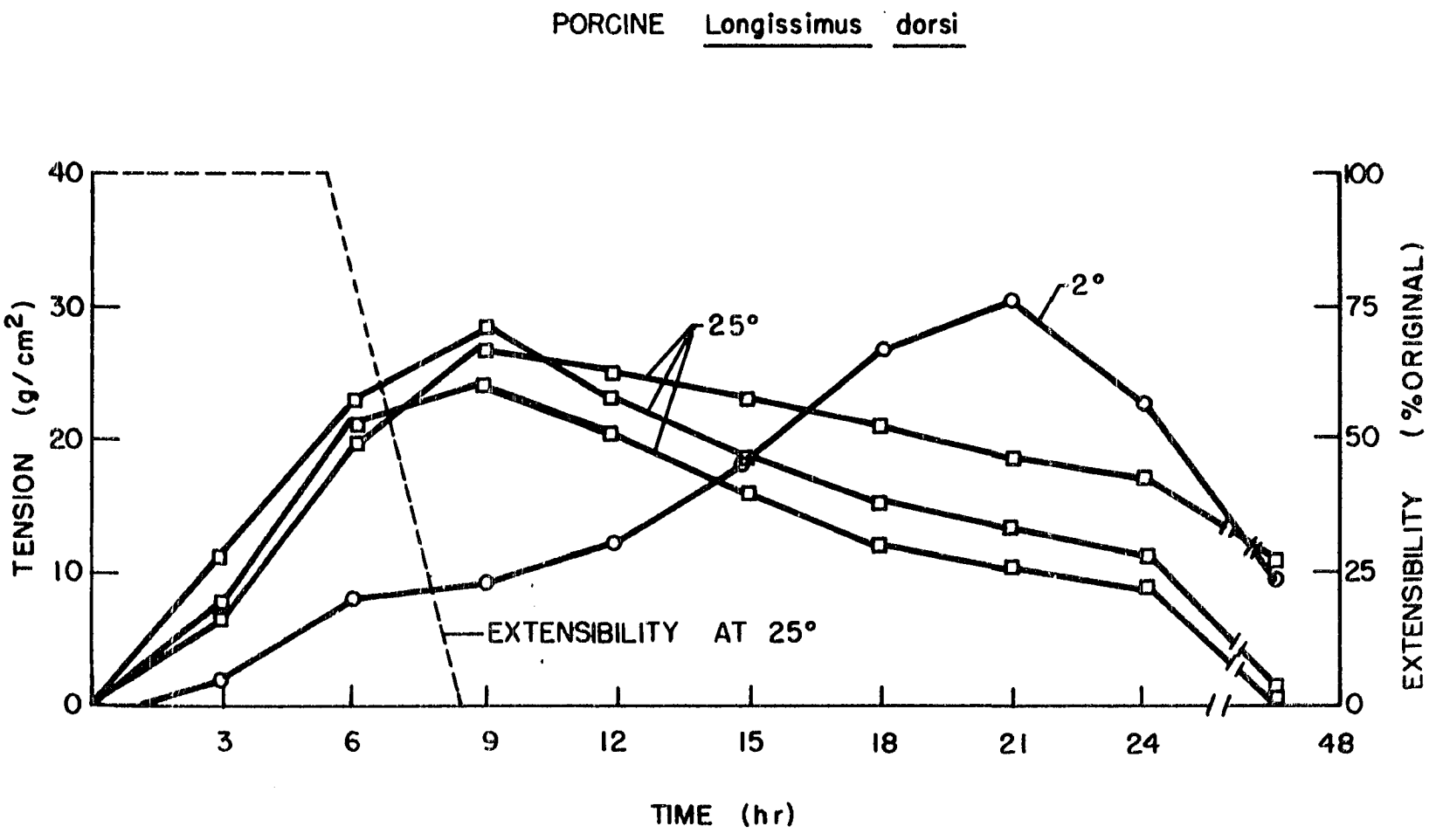
after that time. In this experiment, special care was taken to keep the strip in air in a very moist condition.

Use of a saline solution such as solution B for the study of post-mortem isometric tension development has several advantages in addition to prevention of dehydration. By including NaN_3 in the solution, it was possible to effectively prevent bacterial growth and thereby eliminate any possibility that the loss in ability to maintain isometric tension was due to bacterial degradation of the fiber. Furthermore, use of a bathing medium makes it possible to test the effect of varying external pH, ionic conditions, or chemical environment on post-mortem isometric tension development. Consequently, all isometric tension development experiments described hereafter were done with strips immersed in a solution.

Since most of the work on rigor mortis has used extensibility changes for following development of rigor mortis, a study was done to correlate isometric tension development with changes in post-mortem muscle extensibility. Figure 2 shows that at 25°, porcine longissimus dorsi muscle becomes almost totally inextensible about the same time that it reaches maximum isometric tension development. As is clear from Figure 2, isometric tension development begins before any detectable change in extensibility. Also, this study confirmed the earlier finding that once a muscle becomes inextensible, it does not regain its extensibility, whereas, as shown in Figures 1 and 2, post-mortem muscle does lose its ability to maintain isometric tension.

One of the advantages of using the E and M Physiograph for measuring isometric tension was that five muscle strips from a single animal could

Figure 2. Isometric tension development and extensibility of post-mortem porcine longissimus dorsi muscle strips immersed in solution B at 2^{o} or 25^{o}. All measurements shown are on strips from a single animal, but are typical of results obtained on several animals. The three strips measured at 25^{o} give an indication of the reproducibility obtainable in post-mortem isometric tension measurement.


PORCINE Longissimus dorsi
TENSION (g/cm²)
EXTENSIBILITY (%ORIGINAL)
TIME (hr)
25°
2°
EXTENSIBILITY AT 25°
0
10
20
30
40
0
25
50
75
100
3
6
9
12
15
18
21
24
48

be measured simultaneously. This helped eliminate much of the animal variation encountered in earlier experiments. By use of the E and M Physiograph and external bathing solution, and by taking care that all strips were attached to the myograph under the same initial tension, it was possible to obtain a great deal of reproducibility in tension patterns of different muscle strips from the same animal (Figure 2). In the experiment shown in Figure 2, three strips of porcine longissimus dorsi muscle were removed from the same animal, attached to three separate isometric myographs and subjected to the same environmental temperature. The results show that both the time-course of isometric tension development and decline, and the maximal amount of isometric tension developed was very similar among the three strips. The small variation that does exist is probably due to differences in preparation of the strips and in the manner in which the strips were attached to the transducers. Extreme care was taken in all experiments to minimize these variations, but frequently some unavoidable stretching of the strips occurred.

Since immersion of muscle strips in solution B causes little change in post-mortem isometric tension development but does prevent the dehydration frequently encountered with strips in air, it was considered desirable to reexamine the effect of temperature on post-mortem isometric tension development. It has previously been shown that temperature has a large effect on post-mortem isometric tension development of muscle strips in air. As shown in Figures 3 and 4, porcine and rabbit longissimus dorsi muscle strips immersed in a phosphate-buffered solution exhibit very similar post-mortem isometric tension patterns. In Figures 3 and 4

Figure 3. Effect of temperature on post-mortem isometric tension development of rabbit *longissimus dorsi* muscle. Bathing medium was : 0.06M phosphate buffer, pH 7.2, 0.05M KCl, 5mM $MgCl_2$, 1mM NaN_3. Seven strips measured at 2°, 4 at 16°, 8 at 25°, and 3 at 37°.

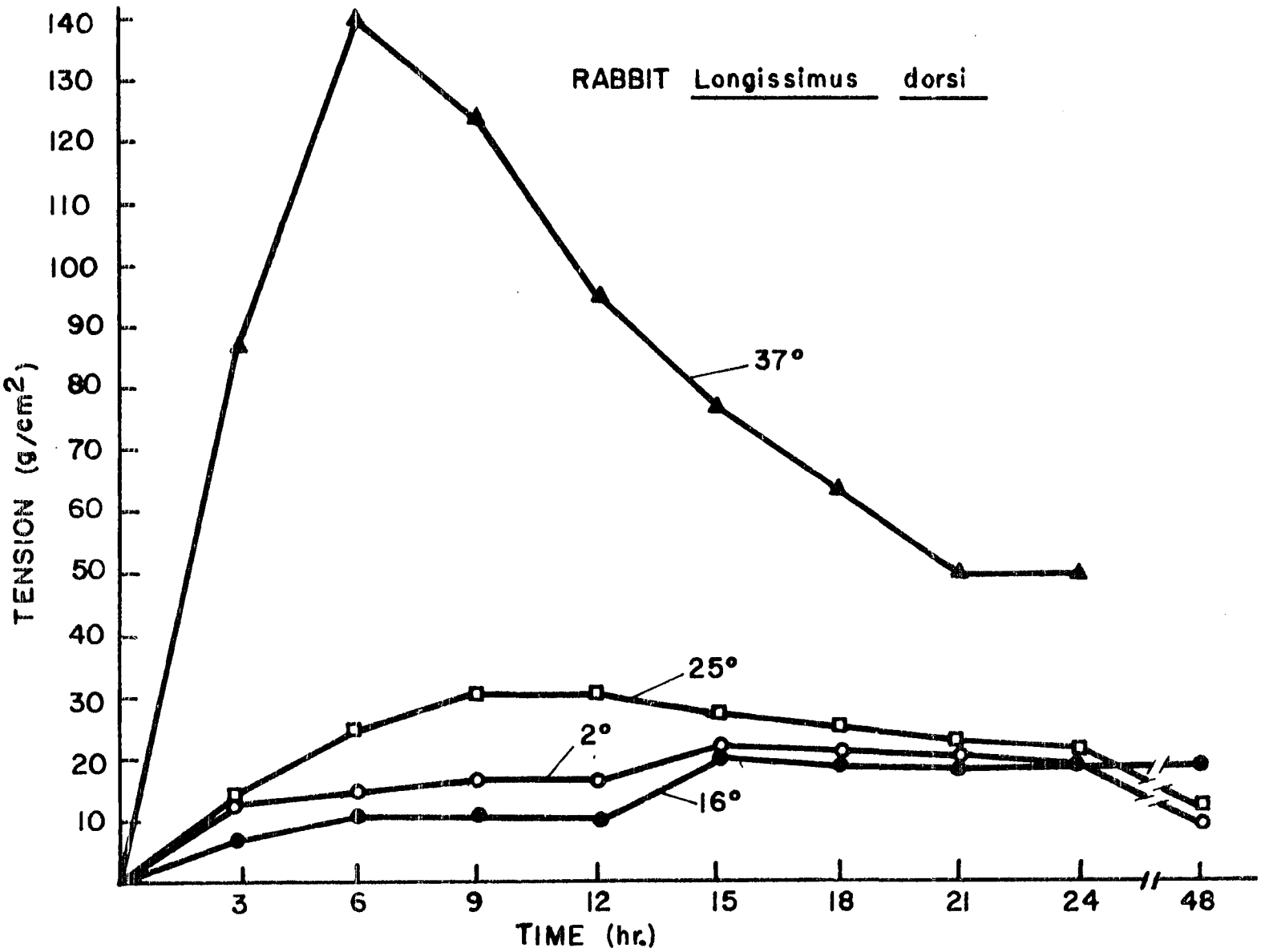
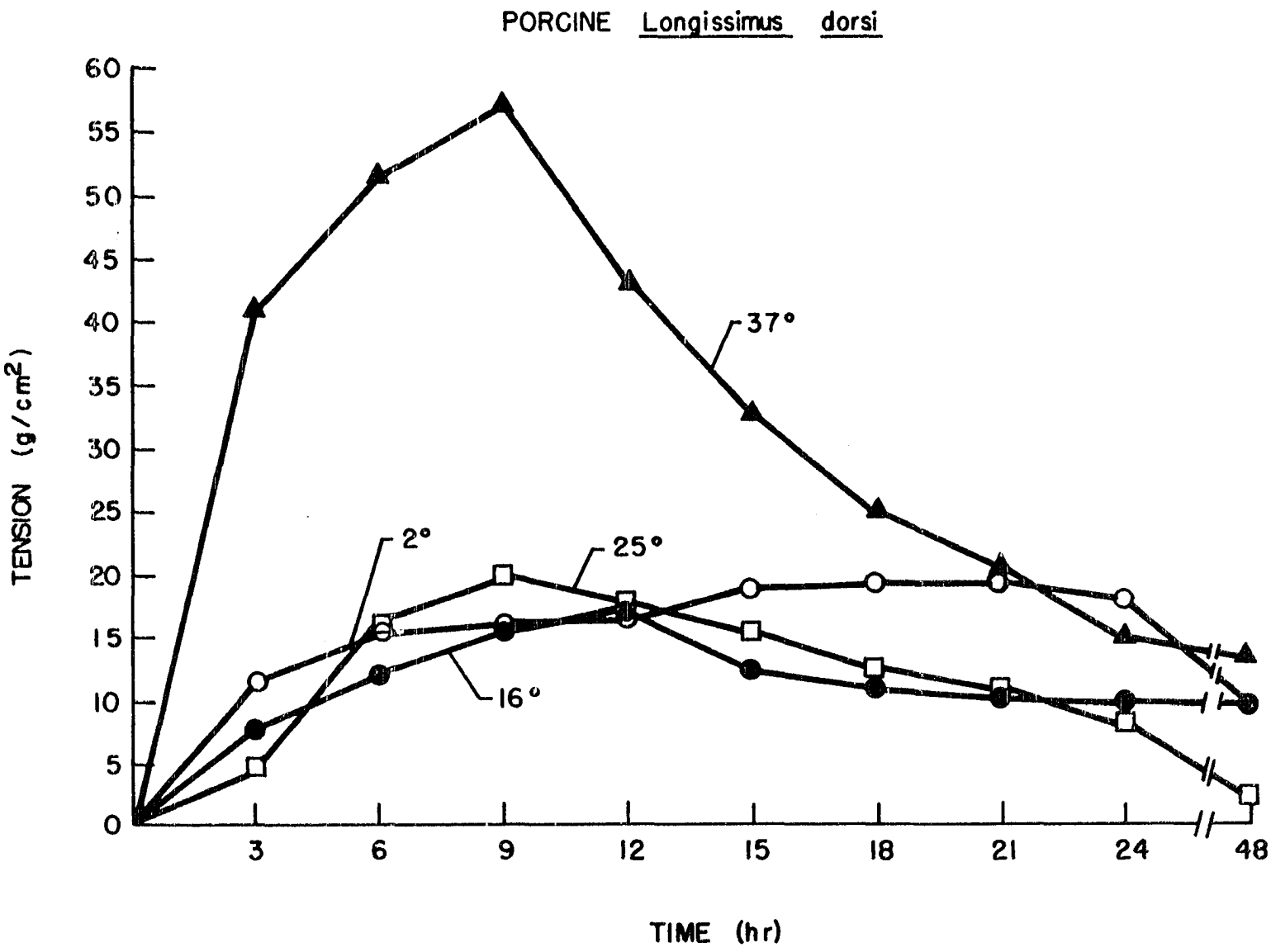

RABBIT Longissimus dorsi
TENSION (g/cm²)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
37°
25°
2°
16°
3
6
9
12
15
18
21
24
48
TIME (hr.)

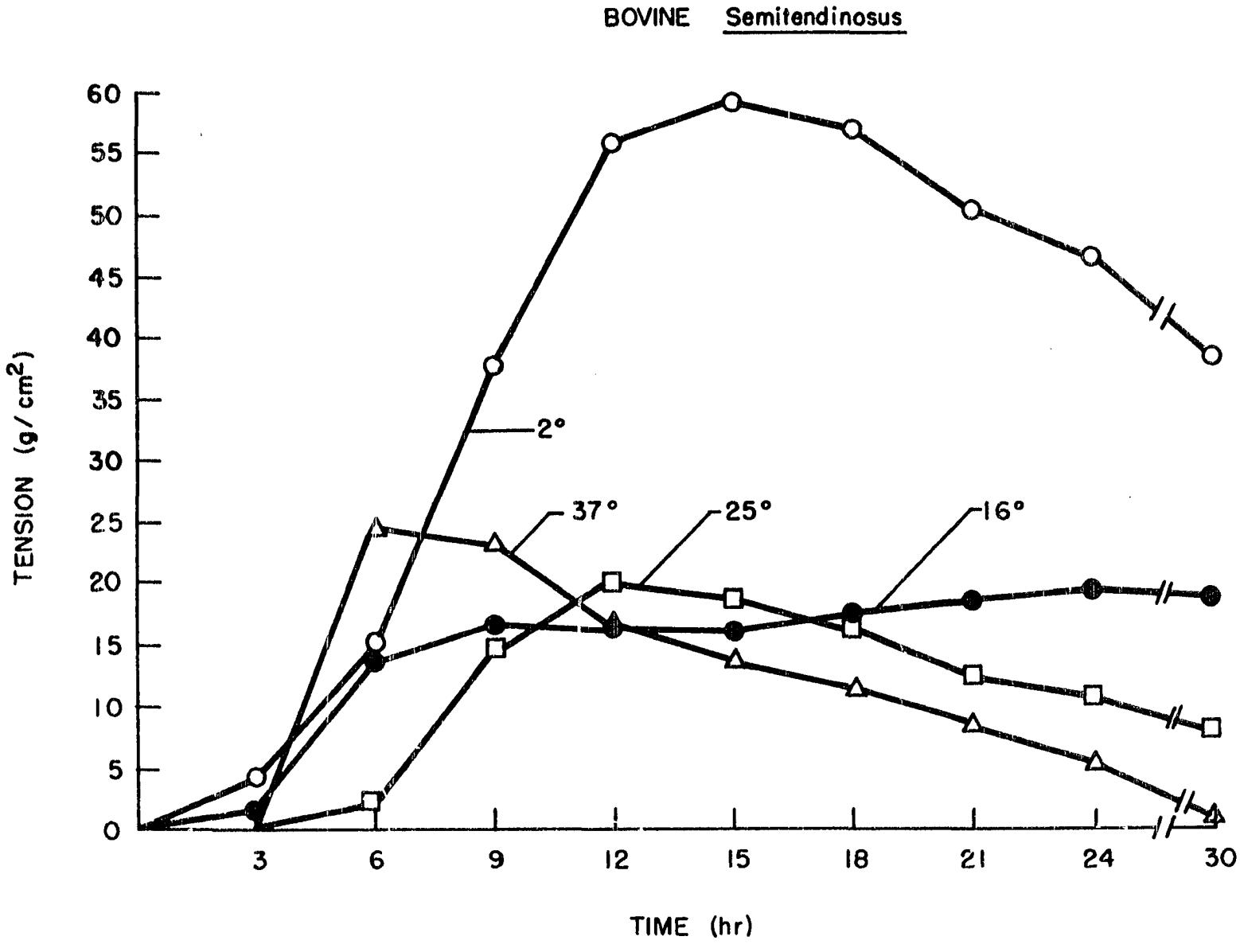
Figure 4. Effect of temperature on post-mortem isometric tension development of porcine *longissimus dorsi* muscle. Bathing medium was: 0.06 M phosphate buffer, pH 7.2, 0.05M KCl, 5mM $MgCl_2$, 1mM NaN_3. Seven strips measured at 2^{o}, 3 at 16^{o}, 8 at 25^{o}, and 6 at 37^{o}.


PORCINE Longissimus dorsi
TENSION (g/cm²)
TIME (hr)
37°
25°
2°
16°
0
5
10
15
20
25
30
35
40
45
50
55
60
3
6
9
12
15
18
21
24
48

and in all following figures presenting tension results, isometric tension is expressed as g/cm^2 of muscle cross-sectional area; this was calculated according to the method of Helander and Thulin (1962). Rabbit and porcine longissimus dorsi muscle develop very little isometric tension at 2, 16, or 25°, but muscle from both species develops considerable tension at 37°. Rabbit muscle at 37° develops much more tension ($140g/cm^2$) than porcine muscle at this temperature ($55g/cm^2$). The effect of temperatures above 16° on post-mortem shortening of rabbit muscle can also be seen at 25° where rabbit muscle develops about 30 g/cm^2 of tension compared to approximately 20 g/cm^2 of tension for porcine muscle at this temperature. The results with porcine muscle isometric tension are in contrast to those of Galloway and Goll (1967) who found that porcine muscle shortened more at 2° than at 16, 25 or 37°. Galloway and Goll's measurements were done on strips in air, and it is possible that dehydration obscured tension development at temperatures above 10° in their experiments.

In contrast to rabbit and porcine muscle, bovine semitendinosus muscle immersed in a phosphate-buffered solution develops maximal tension at 2° (Figure 5), with more than twice as much tension developed at 2° as at 16, 25, or 37°. Although the maximum tension developed by post-mortem bovine muscle is very similar at 16, 25, or 37°, tension development at 37° reaches a maximum sooner than at the other temperatures. In a previous study on bovine strips in air, Busch et al. (1967) found a nine hour difference between initiation of post-mortem tension development at 16° and at 37°. At 37°, the semitendinosus started to develop

Figure 5. Effect of temperature on post-mortem isometric tension development of bovine *semitendinosus* muscle. Bathing medium was: 0.06M phosphate buffer, pH 7.2, 0.05M KCl, 5mM $MgCl_2$, 1mM NaN_3. Four strips were measured at each temperature.


BOVINE Semitendinosus
TENSION (g/cm²)
60
55
50
45
40
35
30
25
20
15
10
5
0
2°
37°
25°
16°
3
6
9
12
15
18
21
24
30
TIME (hr)

tension after 3 hours, whereas it did not begin to develop tension until after 12 hours at 16°. These differences were much smaller in the present study since tension development began after 3 hours at both 16° and 37°. It seems probable that the higher sensitivity of the E and M physiograph used in the present study made it possible to detect the small amount of tension developed between 3 and 6 hours post-mortem at 16°, whereas this small tension development was not detected by the earlier, less sensitive isometer (Jungk et al., 1967).

It is clear from Figures 3, 4, and 5 that when post-mortem isometric tension development is studied on muscle strips immersed in solution, an eventual decline in isometric tension occurs at temperatures above 10° where it had been difficult to detect tension decline on strips measured in air. In fact, at 37° in bovine tissue, there was a complete loss of isometric tension by 30 hours. In porcine and rabbit longissimus dorsi muscle at 37°, the rate of decline was very rapid from 9 to 21 hours post-mortem but then appeared to slow. Between 9 and 21 hours post-mortem, tension decreased to at least one third of maximum tension in both species. Other studies on rabbit psoas muscle indicated that this muscle lost almost all tension development at 37° within 24 hours post-mortem (Figure 8). Rate of tension decline appeared to depend on the maximum amount of tension developed. The greater the tension development, the greater was the rate of tension decline. This may provide an explanation for the observation that tension decline appeared quite variable at 16°, and frequently, it was not possible to detect any tension decline at all during the first 30 hours post-mortem at 16°. However, if the 16° strips were

left long enough (72 hours), some tension decline was noted.

Effect of pH on Isometric Tension of Muscle Strips and ATPase Activity of Myofibrils

The study just described clearly shows that both isometric tension development and loss in ability to maintain isometric tension occur in post-mortem muscle strips. Subsequent studies in this investigation were designed to discover the cause of isometric tension decline. It is well known that a variable decrease in pH is one of the characteristic chemical changes in post-mortem muscle, so an initial study was designed to determine whether pH *per se* would cause post-mortem isometric tension development and decline. As shown in Figure 6, decreasing extracellular pH from 7.0 to 5.0 shortens the time required for initiation of isometric tension development and decline in post-mortem rabbit *psoas* muscle at 37°, but has little effect on the basic tension pattern. It appears that maximal tension development at 37° increases as extracellular pH is increased up to 6.5 but decreases slightly between pH 6.5 and 7.0. Results very similar to these were obtained with porcine *longissimus dorsi* muscle at 37°.

When the effect of pH on isometric tension patterns of rabbit *psoas* at 2° was studied, an entirely different pattern emerged (Figure 7). At 2°, decreasing the extracellular pH from 7.0 to 5.5 decreases the rate of isometric tension development and decline. Maximal tension development was about 23 g/cm^2 at pH 7.0 (within 0.5 hour post-mortem), decreased to 10 g/cm^2 at pH 6.0, and then increased to 24-25 g/cm^2 at pH 5.5 or 5.0. The tension pattern at pH 6.5 or 7.0 was unusual in that tension developed rapidly, reached a maximum after 0.5 hour, and then after three hours

Figure 6. Effect of extracellular pH on post-mortem isometric tension development of rabbit psoas muscle at 37°. The bathing medium pH was adjusted by changing pH of the Tris-acetate buffer in solution B. Points are averages of measurements made on four different animals.

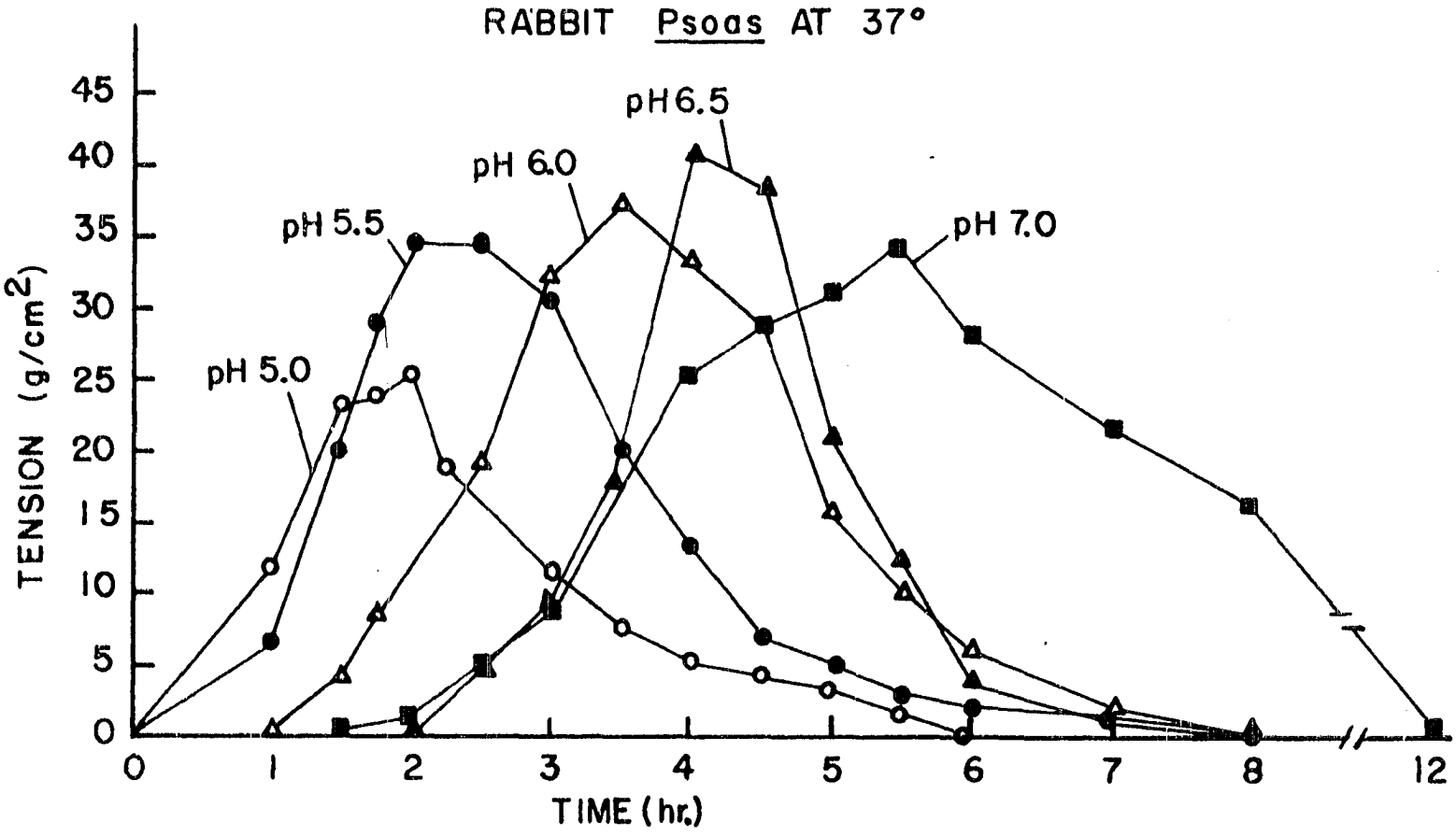
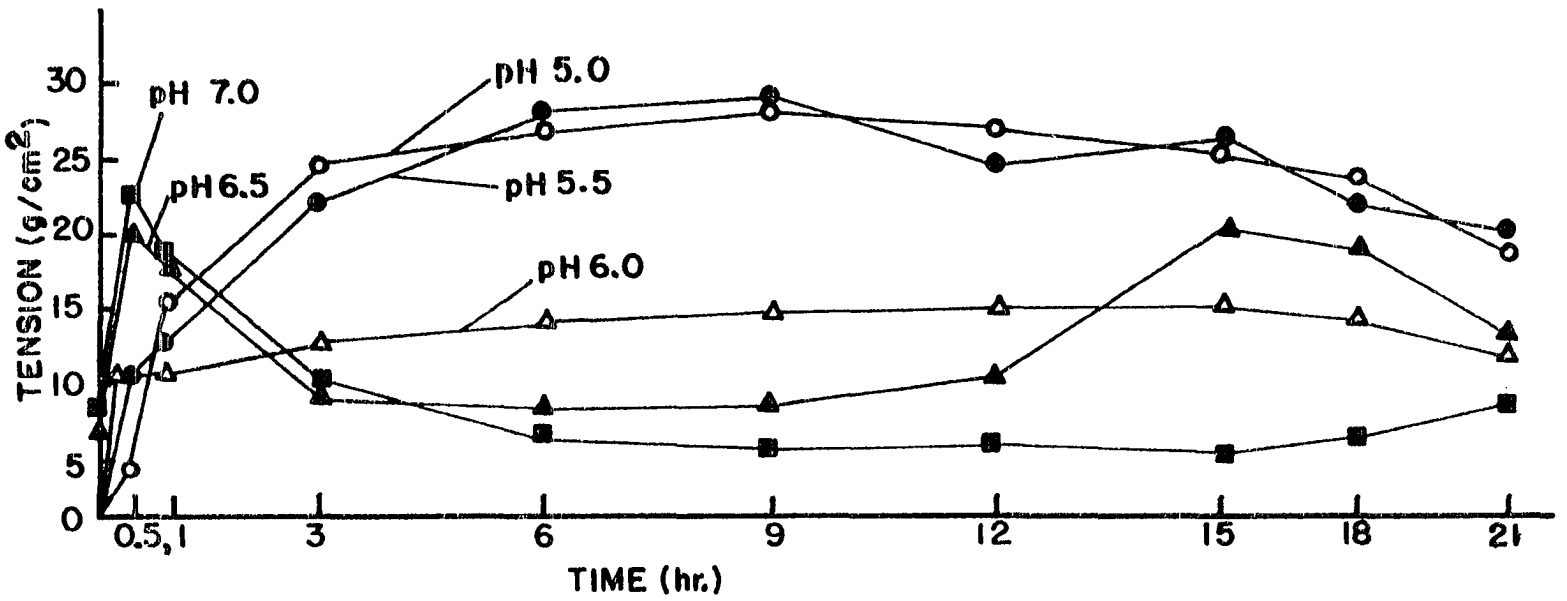

RABBIT Psoas AT 37°
pH 6.5
pH 6.0
pH 5.5
pH 7.0
pH 5.0
TENSION (g/cm²)
TIME (hr.)
0
5
10
15
20
25
30
35
40
45
0
1
2
3
4
5
6
7
8
12

Figure 7. Effect of extracellular pH on post-mortem isometric tension development of rabbit psoas muscle at 2^{o}. The bathing medium pH was adjusted by changing pH of the Tris-acetate buffer in solution B. Points are averages of measurements made on four different animals.

RABBIT Psoas AT 2°



post-mortem, decreased to approximately 50% of maximal tension. This level of tension was maintained for the next 9 hours, after which there was an increase in tension up to 20 g/cm^2 at pH 6.5. The reason for this second development of tension is unknown, but it has been observed in several patterns of 2° rabbit muscle at pH 6.5. It is interesting to note that the second increase in tension occurs in the absence of ATP.

These results suggest that extracellular pH does not play a major role in the development of post-mortem tension. It is possible, however, that impermeability of the cell membrane may be preventing intracellular pH changes, and that intracellular pH is important in rigor mortis. It is known that extracellular pH affects membrane permeability, and the changes observed in this study may therefore be due in part to permeability effects. However, it is difficult to believe that the large extracellular pH differences used in this study did not also cause at least some changes in intracellular pH of the fibers. At any rate, it is clear that low extracellular pH does not itself cause the decline of post-mortem isometric tension development.

To obtain some information on possible effects of intracellular pH on myofibrils, a study was done to determine the effects of storing myofibrils, prepared immediately after death, in a saline solution at different pH values. The ATPase activity of the myofibrils was used as a measure of any structural or conformational change caused by the storage conditions. Tables 1, 2, and 3 show that the myofibrillar ATPase activity of porcine myofibrils is not affected by storage at 2° for periods up to 7 days and at pH values ranging from 5.5 to 8.0. The Ca^{++}-modified

Table 1. Effect of pH on Ca^{++}-modified ATPase activity of porcine myofibrils[a]

	pH					
	5.5	6.0	6.5	7.0	7.5	8.0
0 hr	0.313±0.025[b]	0.313±0.025	0.313±0.025	0.313±0.025	0.313±0.025	0.313±0.025
24 hr	0.344± 0.028	0.342±0.024	0.323±0.029	0.329±0.027	0.343±0.030	0.336±0.030
72 hr	0.292±0.021	0.282±0.014	0.305±0.011	0.309±0.015	0.307±0.009	0.295±0.005
7 days	0.286±0.014	0.299±0.010	0.293±0.007	0.297±0.011	0.342±0.009	0.326±0.014

[a]Conditions of assay: 0.40-0.50 mg protein/ml, 5 mM ATP, 5 mM Ca^{++} in 0.045 M KCl, 0.05 M Tris, pH 7.0.

[b]Means plus or minus standard errors of six determinations.

Table 2. Effect of pH on Mg^{++} modified ATPase activity of Porcine Myofibrils[a]

	pH					
	5.5	6.0	6.5	7.0	7.5	8.0
0 hr	0.240±0.033[b]	0.240±0.033	0.240±0.033	0.240±0.033	0.240±0.033	0.240±0.033
24 hr	0.249±0.027	0.252±0.025	0.217±0.023	0.244±0.023	0.269±0.035	0.254±0.025
72 hr	0.201±0.019	0.191±0.013	0.213±0.028	0.188±0.016	0.190±0.008	0.209±0.019
7 day	0.220±0.016	0.227±0.014	0.207±0.007	0.214±0.008	0.223±0.015	0.214± 0.003

[a]Conditions of assay: 0.40-0.50 mg protein/ml, 5 mM ATP, 5 mM Mg^{++}+ 0.1 mM Ca^{++}, 0.045 M KCl, 0.05 M Tris, pH 7.0.

[b]Means plus or minus standard errors of six determinations.

Table 3. Effect of pH on Mg-modified ATPase activity of porcine myofibrils in the presence of EGTA[a]

	pH					
	5.5	6.0	6.5	7.0	7.5	8.0
0 hr	0.036±0.005[b]	0.036±0.005	0.036±0.005	0.036±0.005	0.036±0.005	0.036±0.005
24 hr	0.034±0.003	0.042±0.002	0.038±0.004	0.036±0.004	0.047±0.004	0.052±0.005
72 hr	0.019±0.003	0.021±0.003	0.033±0.006	0.027±0.003	0.028±0.001	0.030±0.004
7 day	0.021±0.003	0.025±0.004	0.023±0.005	0.030±0.006	0.032±0.004	0.040±0.006

[a]Conditions of assay: 0.40-0.50 mg protein/ml, 5 mM ATP, 5 mM Mg^{++} + 1 mM EGTA, 0.045 M KCl, 0.05 M Tris, pH 7.0.

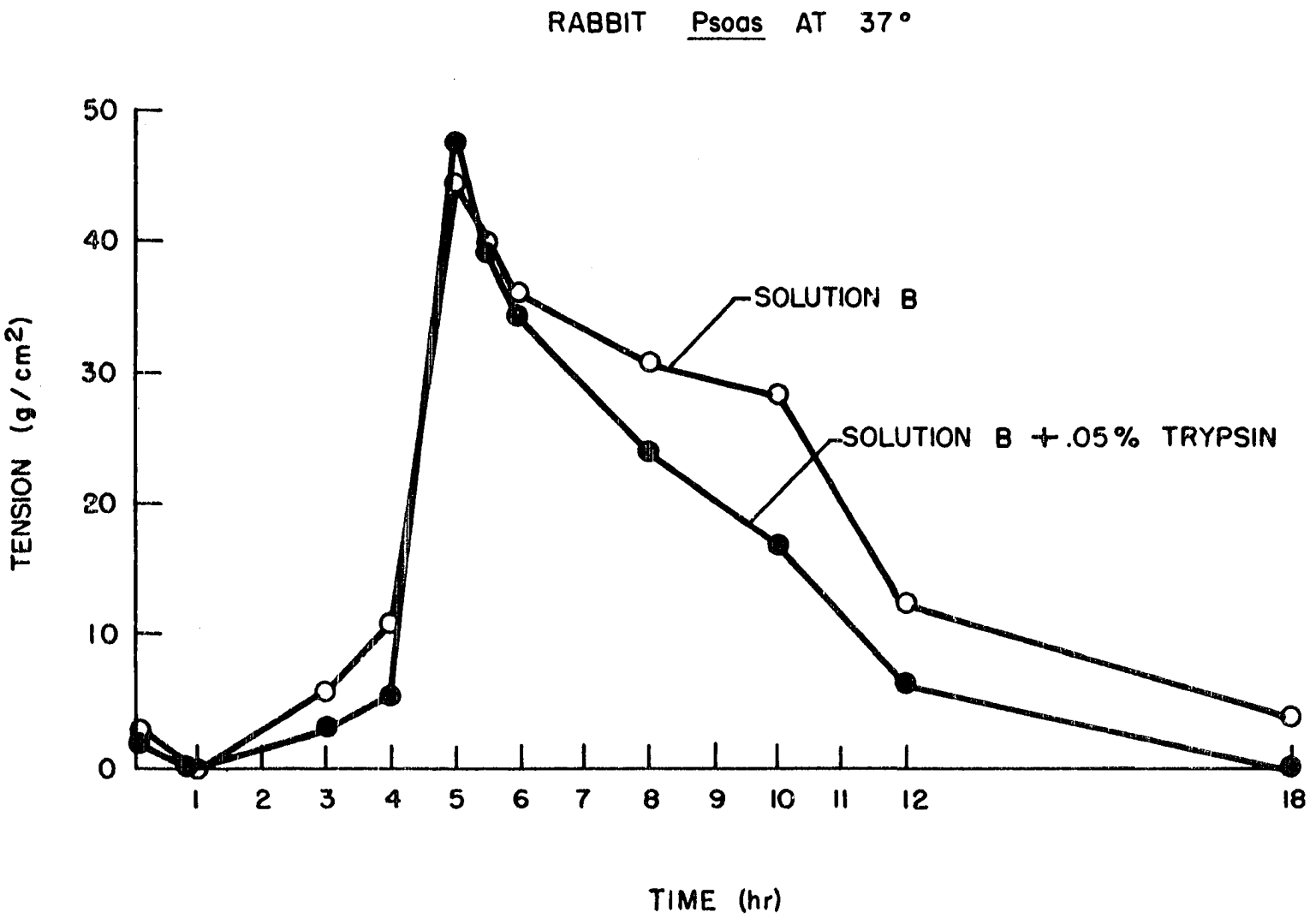
[b]Means plus or minus standard errors of six determinations.

ATPase activity of myofibrils is a measure of integrity of the myosin molecule itself, the Mg^{++}-modified ATPase activity is a measure of the nature of the actin-myosin interaction, and the Mg^{++}-EGTA-modified ATPase activity is a measure of integrity of the tropomyosin-troponin complex. Consequently, the results of this study clearly show that variations in pH from 5.5 to 8.0 do not influence any of these myofibrillar properties during the first 7 days post-mortem. This provides some support for the conclusion that changes in intracellular pH are not responsible for the decline in post-mortem isometric tension development.

Effect of Trypsin on Muscle Strips

It has been widely believed that intracellular cathepsins, presumably released from lysosomes, play an important role in post-rigor softening of carcasses. Although it has been difficult to obtain any unequivocal evidence that cathepsins are indeed active in post-morten muscle, it nevertheless seemed possible that post-mortem isometric tension decline was due to a very limited catheptic proteolysis, undetectable by ordinary methods. Consequently, to test this possibility, the isometric tension pattern of rabbit psoas muscle was examined in the presence and absence of trypsin added to the bathing solution (solution B). Stromer et al. (1967) have shown that very brief treatment of myofibrils with trypsin caused removal of M- and Z-lines. Therefore, it was anticipated that addition of trypsin to the bathing solution would either inhibit tension development completely or would cause almost instantaneous release of tension. However, as shown in Figure 8, trypsin had no clear effect on

Figure 8. Effect of trypsin on post-mortem isometric tension development of rabbit psoas muscle at 37°. In each experiment two strips were placed in solution B and two other strips from the same animal were placed in solution B plus 0.05% (v/w) trypsin. Results are averages from two animals.


RABBIT Psoas AT 37°
TENSION (g/cm2)
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
18
TIME (hr)
SOLUTION B
SOLUTION B + .05% TRYPSIN

the post-mortem isometric tension pattern. These results suggested that trypsin was unable to penetrate the cell membrane, so additional experiments were done with 1mM deoxycholate added to the bathing medium to cause rupture of the sarcolemma. However, even in the presence of deoxycholate, trypsin had no effect on the isometric tension pattern. Assays with a synthetic substrate showed that the added trypsin was still active after 8 hours at 37° in the presence of deoxycholate. It is difficult to believe that trypsin would not cause a drastic decrease in isometric tension development if it were in contact with the myofibrils. Therefore, these results indicate that the sarcolemma is not permeable to trypsin even after 8 hours at 37° in the presence of deoxycholate. This in turn suggests that catheptic enzymes present in leucocytes probably do not have much effect on myofibrils during the onset and resolution of rigor mortis, since the cell membrane presumably would not be any more permeable to these enzymes than it apparently is to trypsin.

Effect of Divalent Cations on the Isometric Tension Pattern

Greaser et al. (1967) reported that the sarcoplasmic reticulum loses its Ca^{++} accumulating ability approximately the same time that shortening occurs in post-mortem muscle. This finding suggests that release of Ca^{++} from sarcoplasmic reticular membranes initiates post-mortem tension development. Consequently, Ca^{++} was added to the external bathing medium of post-mortem muscle fibers in anticipation that this would shorten the time required for development of isometric tension. In order to make valid

comparisons, 1mM EDTA was added to the external bathing medium of the control strips, since Ca^{++} is a contaminant of most reagents including distilled water. Various levels of Ca^{++} from 0.1mM to 10mM were tested in initial experiments, but 1mM Ca^{++} was finally selected for use in subsequent experiments since this level gave results similar to those found at the higher levels. Quite surprisingly, addition of 1mM Ca^{++} had little effect on the time at which isometric tension development began, but there was a very striking difference between the Ca^{++}-treated strip and the EDTA-control strip (Figure 9) in rate of tension release. Once the Ca^{++}-treated strip has developed maximum tension, there is a very rapid release of tension, and after 9 to 12 hours post-mortem, tension development has decreased nearly to zero. In the presence of EDTA, post-mortem muscle strips develop more tension than in the presence of Ca^{++}, and once maximum tension development has been attained, this level is maintained with very little loss, even after 24 hours post-mortem.

Both Ca^{++} and Mg^{++} are obligatory requirements for contraction of muscle fibers, and since the EDTA-treated fibers were clearly developing considerable isometric tension, it seems certain that the added EDTA is not penetrating the cell membrane and chelating intracellular Mg^{++} or Ca^{++}. Consequently, various detergents were added to the bathing medium to attempt to rupture the sarcolemmal membranes, or at least increase their permeability to EDTA. In order to ascertain that detergents would not cause effects that would mask the Ca^{++} and EDTA effects, a control experiment was done in which 1mM deoxycholate was added to the bathing solution (solution B). Figure 10a shows that at 37°, deoxycholate

Figure 9. Effect of extracellular Ca^{++} and EDTA on post-mortem isometric tension development of rabbit psoas at 37^{o}. Strips were immersed in solution B plus 1mM Ca^{++} or solution B plus 1mM EDTA. When EDTA was added, the 5mM $Mg\ Cl_2$ was omitted from solution B. Each point is the average of measurements made on two strips from the same muscle.

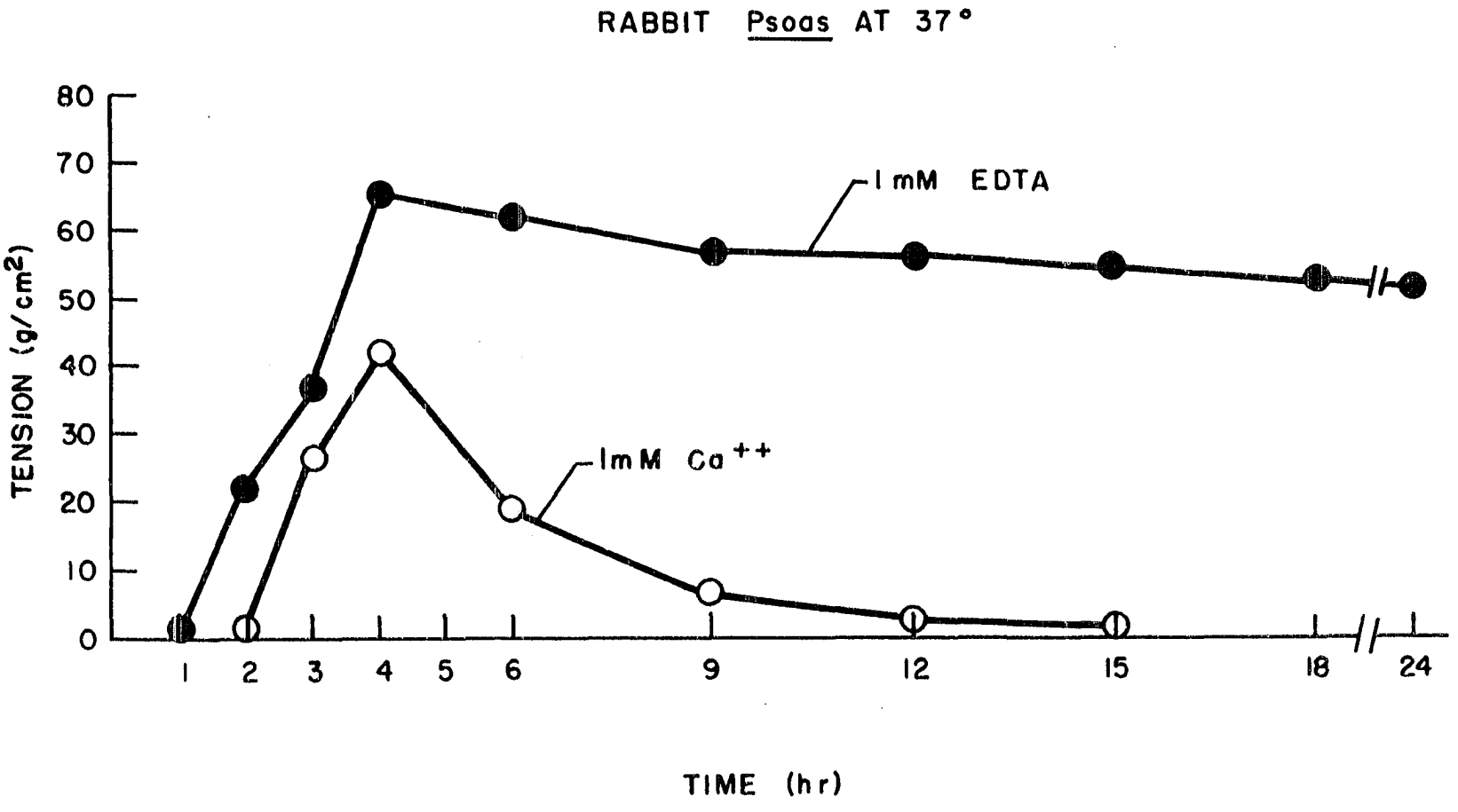
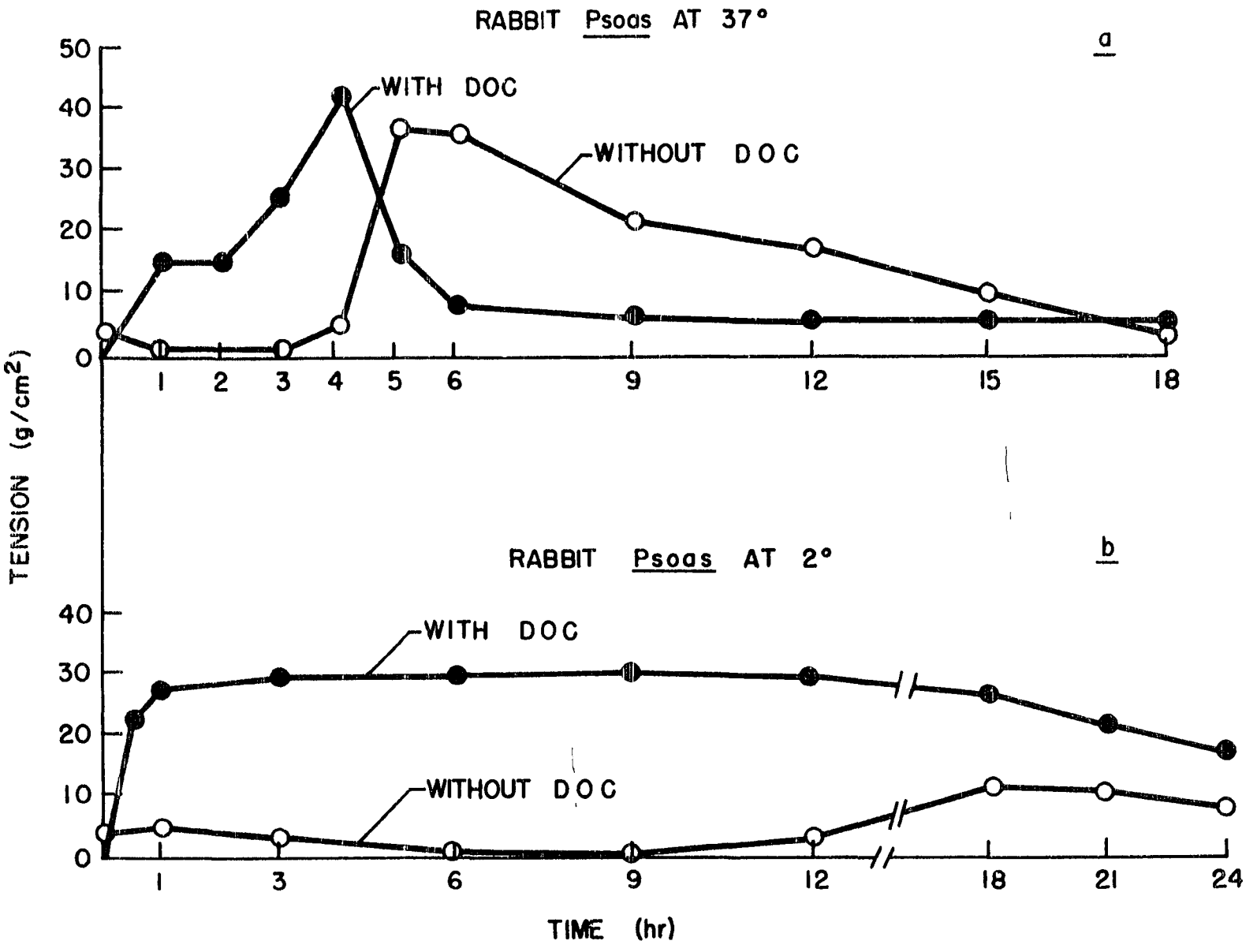

RABBIT Psoas AT 37°
TENSION (g/cm²)
80
70
60
50
40
30
20
10
0
1 mM EDTA
1mM Ca++
1
2
3
4
5
6
9
12
15
18
24
TIME (hr)

Figure 10a. Effect of the addition of 1mM deoxycholate to solution B on post-mortem isometric tension development of rabbit psoas muscle at 37°. Points are averages of measurements made on four strips from two different animals.

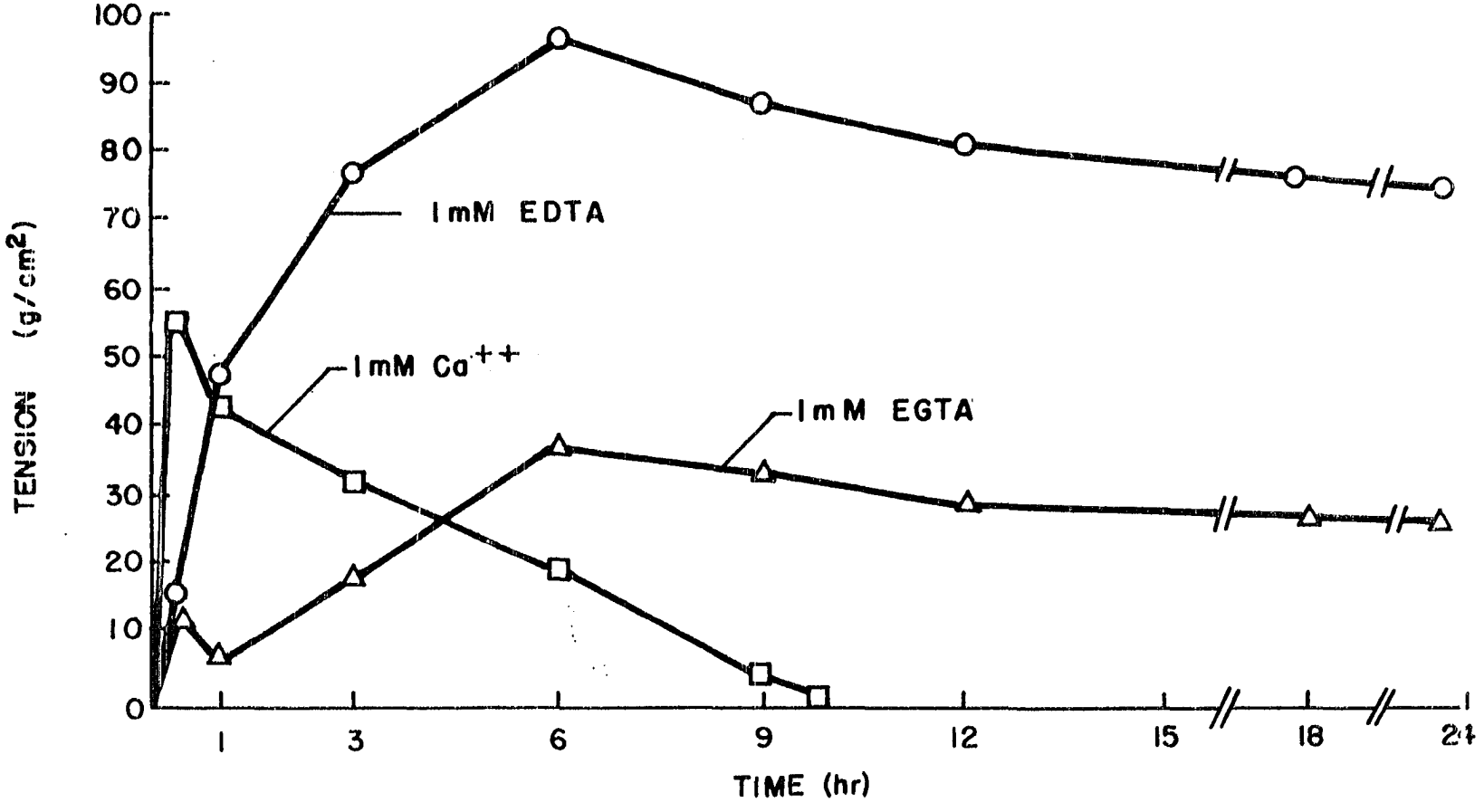
Figure 10b. Effect of the addition of 1mM deoxycholate to solution B on post-mortem isometric tension development of rabbit psoas muscle at 2°. Points are averages of measurements made on four strips from two different animals.


RABBIT Psoas AT 37°
a
WITH DOC
WITHOUT DOC
50
40
30
20
10
0
1
2
3
4
5
6
9
12
15
18
TENSION (g/cm²)
RABBIT Psoas AT 2°
b
WITH DOC
WITHOUT DOC
40
30
20
10
0
1
3
6
9
12
18
21
24
TIME (hr)

simply shortens the time required for initiation of isometric tension development and increases the rate of tension loss once maximum tension has been reached. Since the sarcoplasmic reticulum binds most of the intracellular Ca^{++} in muscle, a possible explanation for this result is that added deoxycholate disrupts the Ca^{++}-sequestering ability of the sarcoplasmic reticulum, and the Ca^{++} released from the disrupted sarcoplasmic reticulum causes a very rapid tension development and decline. At 2^{o}, the presence of deoxycholate appears to increase maximum isometric tension development quite significantly (Figure 10b), but it should be pointed out that the control strips in this comparison developed less tension than normal rabbit psoas at 2^{o} (cf. Figure 3).

Thus, these control studies suggest that addition of 1mM deoxycholate to solution B causes increased permeability of the sarcolemma and also results in loss of the Ca^{++}-sequestering ability of the sarcoplasmic reticulum. Consequently, it was surprising to find that when the effects of added Ca^{++} or EDTA on post-mortem isometric tension development were studied in the presence of 1mM deoxycholate (Figure 11), the results were quite similar to those found previously in the absence of deoxycholate (cf. Figures 9 and 11). As shown in the control experiments (Figure 10), addition of deoxycholate shortened the time required for tension initiation in all treatments. It was also noticed in this experiment that addition of solution B containing both 1mM Ca^{++} and 1mM deoxycholate to the isometrically suspended fibers caused a very rapid tension development and decline. This tension development and decline was usually completed within the first ten minutes after addition of the deoxycholate-Ca^{++}

Figure 11. Effect of Ca^{++}, EDTA, and EGTA on post-mortem isometric tension development of rabbit psoas at 25°. Bathing media were: solution B plus 1mM Ca^{++}; solution B plus 1mM EGTA; solution B (without Mg Cl_2) plus 1mM EDTA. All solutions contained 1mM deoxycholate. Measurements were taken from a single animal.

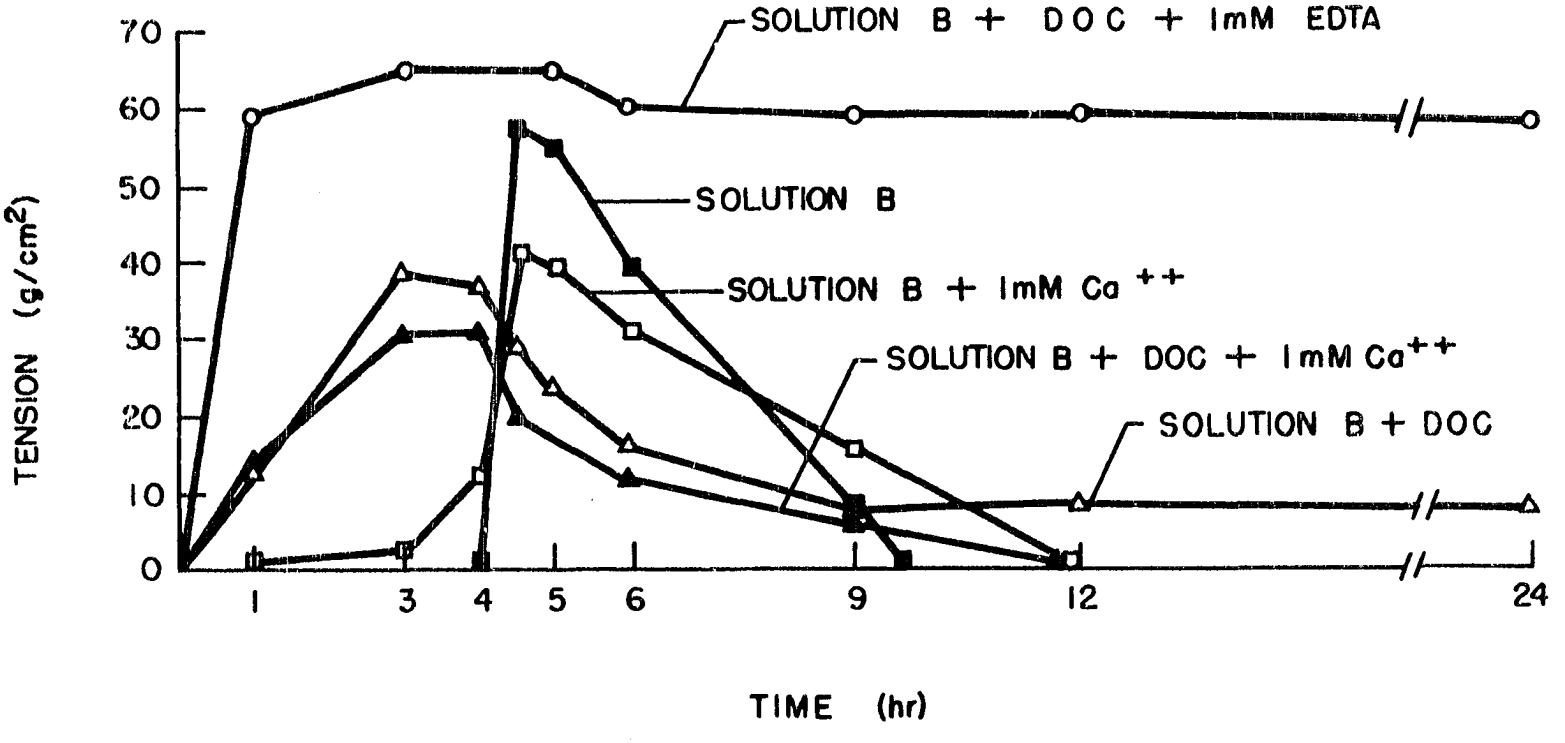

RABBIT Psoas AT 25°
TENSION (g/cm²)
100
90
80
70
60
50
40
30
20
10
0
1mM EDTA
1mM Ca++
1mM EGTA
1
3
6
9
12
15
18
24
TIME (hr)

solution. Because of its very rapid nature and because it occurred only when deoxycholate and Ca^{++} were both present in solution B, this rapid tension development and decline was probably not associated with the normal post-mortem isometric tension pattern. Consequently, the rapid, Ca-plus-deoxycholate-induced tension development and decline is not included in Figure 11 nor (with the exception of Figure 14) in any of the other figures where it occurred in this study (Figures 12 and 17). Omitting this rapid tension development and decline also helps simplify and clarify interpretation of the post-mortem isometric tension patterns.

It is obvious in Figure 11 that the muscle strip bathed in the EDTA-containing solution developed even greater tension in the presence of EDTA than it had in its absence (cf. Figures 9 and 11), and furthermore, it maintained this tension without any detectable decline for 24 hours post-mortem. The Ca^{++}-treated strip also developed slightly more isometric tension in the presence of deoxycholate than it had in its absence, and the Ca^{++}-treated strip again exhibited a rapid decline in isometric tension development.

In the experiment shown in Figure 11, a third strip was bathed in solution B plus 1mM EGTA and 1mM deoxycholate. The EGTA-treated strip developed about one third as much tension as the EDTA-treated strip, but otherwise the tension patterns of the EDTA-treated and the EGTA-treated strips were very similar. Since EGTA chelates Ca^{++} but not Mg^{++} and since the solution B plus EGTA combination contained 5mM $MgCl_2$, it is clear that the rapid decline of post-mortem isometric tension is due to Ca^{++} and not to Mg^{++}.

Figure 12. Effect of deoxycholate, Ca^{++}, Ca^{++} plus deoxycholate, and Solution B EDTA plus deoxycholate on post-mortem isometric tension development of rabbit psoas muscle at 37°. Bathing media were: solution B alone; solution B plus 1mM Ca^{++}; solution B plus 1mM deoxycholate; solution B plus 1mM Ca^{++} and 1mM deoxycholate; solution B (without 5mM Mg Cl_2) plus 1mM EDTA plus 1mM deoxycholate. Measurements were made on strips from one animal.


RABBIT Psoas AT 37°
SOLUTION B + DOC + ImM EDTA
SOLUTION B
SOLUTION B + ImM Ca ++
SOLUTION B + DOC + ImM Ca++
SOLUTION B + DOC
TENSION (g/cm2)
70
60
50
40
30
20
10
0
1
3
4
5
6
9
12
24
TIME (hr)

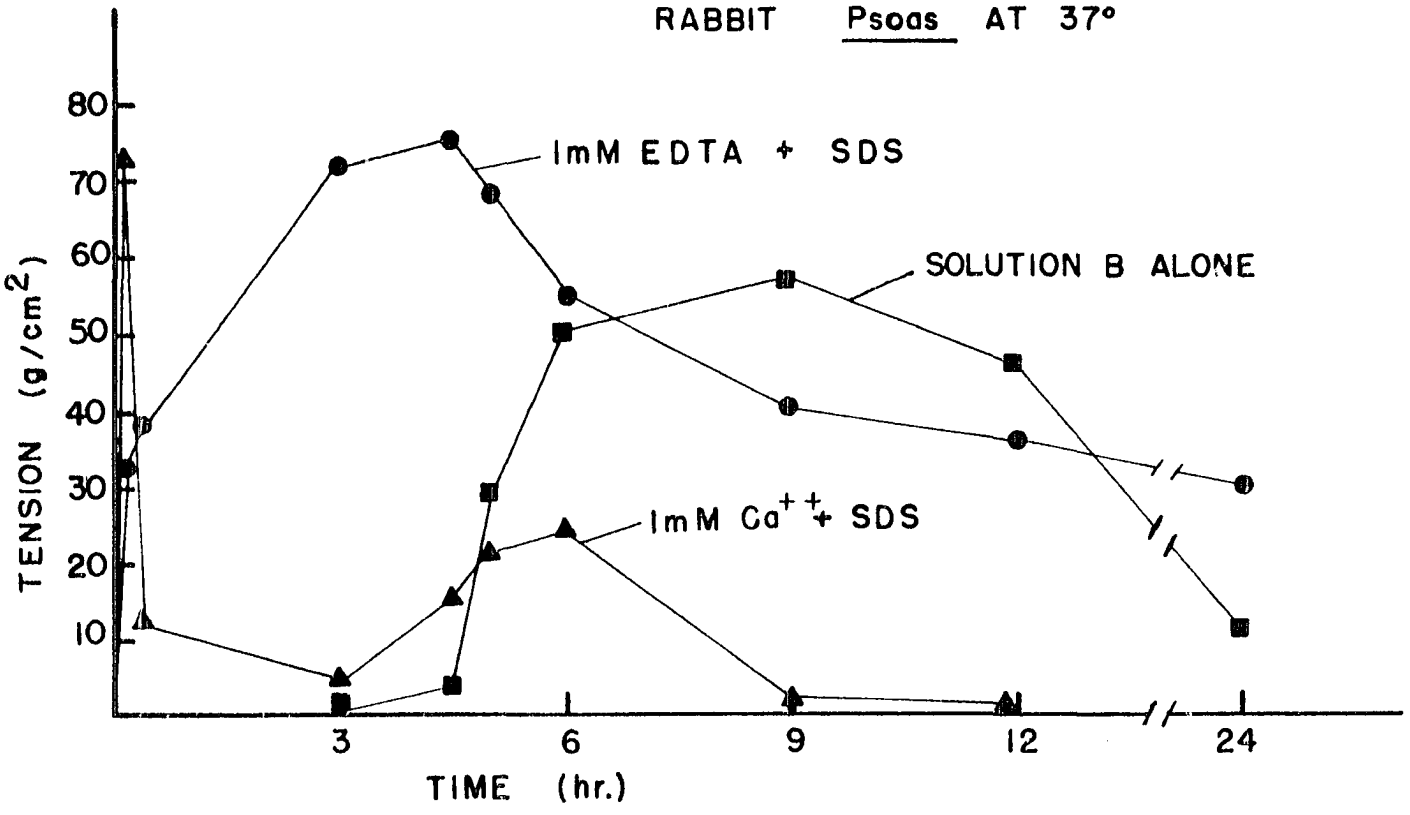
Animal differences can cause some variation in isometric tension patterns, so the preceding conclusions were confirmed by testing the different Ca^{++}, EDTA, and deoxycholate combinations on strips from one animal (Figure 12). The agreement between this experiment and the ones shown in Figures 9 and 11 was excellent. Again, addition of Ca^{++} causes a rapid decline of isometric tension, either in the presence or absence of 1mM deoxycholate, whereas addition of 1mM EDTA in the presence of deoxycholate results in almost no tension decline after 24 hours post-mortem. Since muscle strips contain Ca^{++} in their sarcoplasmic reticular membranes and since there is contaminating Ca^{++} in the solution bathing the strip, isometric tension decline is very rapid in the presence of solution B alone.

Because muscle strips bathed in solution B plus 1mM EDTA and 1mM deoxycholate developed considerable isometric tension, it appeared that EDTA, even in the presence of deoxycholate, was not penetrating the sarcolemmal membrane until after 5-6 hours post-mortem, when tension development had already occurred. It also seemed probable that after 5-6 hours post-mortem, EDTA was able to exert some effect directly on the myofibrils since addition of EDTA resulted in essentially no loss of isometric tension once maximum tension was reached. This suggests that sarcolemmal membrane degradation may occur after 5-6 hours post-mortem and EDTA may then penetrate to the myofibrils. Furthermore, EDTA must have some immediate effect on the muscle fiber, possibly a depolarization of the cell membrane, since the presence of EDTA appears to actually potentiate a rapid isometric tension development. In view of these considerations, it was decided to test the effects of EDTA on post-mortem isometric tension development in

presence of a detergent other than deoxycholate. Hence, an experiment was done using 0.05% SDS (0.05% SDS = 1.7mM) in the bathing medium instead of 1mM deoxycholate (Figure 13). This concentration of SDS has a slightly different effect on permeability than 1mM deoxycholate. Thus, addition of Ca^{++} in the presence of 0.05% SDS caused a very rapid and strong contraction immediately after the strips were placed in this solution. This tension development lasted only five to ten minutes and then decreased rapidly almost to zero. This rapid tension development and decline is similar in time-course but much greater in magnitude than the rapid Ca^{++}-plus deoxycholate-induced tension pattern described for Figure 11. Three hours later, the Ca^{++}-plus-SDS-treated strips developed a small amount of additional tension (rigor tension). Even with 0.05% SDS in the bathing medium, considerable tension development occurred in the presence of EDTA. However, this tension was not maintained at a constant level with increasing time post-mortem, but declined slowly. This slow decline may be due to partial dissolution of the myofibril by the 0.05% SDS.

Since it seemed possible to obtain post-mortem isometric tension development in the presence of either extracellular Ca^{++} or extracellular EDTA, a preliminary study was done attempting to compare isometric tension development with isotonic shortening in post-mortem muscle. These experiments used Ca^{++} in the external bathing solution because it was of interest to find whether the rapid isometric tension decline in the presence of Ca^{++} might be paralleled by a rapid lengthening of isotonically shortened fibers. The very rapid Ca^{++}-plus-deoxycholate-induced tension development and

Figure 13. Effect of sodium dodecyl sulfate (SDS) on post-mortem isometric tension development of rabbit psoas at 37°. Bathing media were: solution B alone; solution B plus 1mM Ca^{++} and 0.05% SDS; solution B (without $MgCl_2$) plus 1mM EDTA and 0.05% SDS. Points are averages of measurements made on strips from two animals.


RABBIT Psoas AT 37°
1mM EDTA + SDS
SOLUTION B ALONE
1mM Ca++ + SDS
TENSION (g/cm2)
80
70
60
50
40
30
20
10
3
6
9
12
24
TIME (hr.)

decline which was described earlier for Figure 11 has been included in Figure 14 to show the close parallel that existed between isometric tension development and isotonic shortening. As shown in Figure 14, the Ca^{++}-plus-deoxycholate-induced tension development and decline is completed within the first 10 minutes post-mortem. This was followed about 2.5 hours later by the normal post-mortem tension development and decline. The isotonic shortening of strips taken from the same animal and exposed to the same environmental conditions closely paralleled the isometric tension development pattern (Figure 14). Thus, the fibers shortened quickly when first exposed to solution B containing 1mM Ca^{++} and 1mM deoxycholate. This was followed by a slow lengthening and then a second shortening corresponding to the second isometric tension development (Figure 14). The second shortening phase was followed by a slow, gradual, lengthening phase. In general, it was noticed that changes in length of the isotonically suspended fibers were much less than the isometric tension changes. Hence, a 50% increase in isometric tension development was paralleled by only a 4.0 to 5.0% change in length of the isotonically suspended fibers. Moreover, although isometric tension decline occurred fairly rapidly and returned nearly to zero after 4 hours post-mortem, the corresponding lengthening of isotonically suspended fibers occurred much more slowly, and the isotonically suspended strips did not return to their initial lengths. Consequently, it appears that isometric tension is probably a more convenient and sensitive method for monitoring post-mortem changes in muscle than is shortening of isotonically suspended strips. Three isometrically suspended strips and two isotonically suspended strips

were included in each experiment in this study, and two of the isometrically suspended and two of the isotonically suspended strips were removed at the points indicated in Figure 14 by the arrows. These strips were used for microscopic studies which will be described later in this section.

All previous experiments testing the effects of Ca^{++} and EDTA on post-mortem isometric tension development had been done with rabbit psoas muscle at 25° or 37°. It was previously shown (cf. Figure 5) that isometric tension development in post-mortem bovine muscle is very different from that in post-mortem rabbit muscle. Thus, tension development in bovine muscle is maximal at 2°, whereas it is minimal at 2° in rabbit muscle. Consequently, the effect of Ca^{++} was studied on isometric tension development in bovine semitendinosus at 2°. As is evident from Figure 15, Ca^{++} had a slightly different effect on isometric tension development in bovine semitendinosus muscle at 2° than it had on rabbit psoas muscle at 37°. Hence, although the addition of Ca^{++} in the presence of deoxycholate causes tension development to begin within the first hour post-mortem, and results in a rapid tension decline in both bovine and rabbit muscle, isometric tension development in bovine muscle at 2° was greater in the presence of Ca^{++} than in the presence of 1mM EDTA. This is the opposite of the situation in rabbit psoas muscle at 37°. However, the presence of 1mM EDTA prevented the decline of isometric tension in bovine muscle at 2°, just as it had in rabbit muscle at 37°. The isometric tension pattern shown for bovine semitendinosus at 2° in solution B alone (Figure 15) is atypical since bovine muscle in solution B at 2° normally developed 2-3

Figure 14. Comparison of isometric tension development and isotonic shortening of rabbit psoas muscle at 37°. Bathing medium was solution B plus 1mM Ca^{++} and 1mM deoxycholate. All measurements were made on one animal. Arrows indicate time at which strips were removed for electron microscopy.

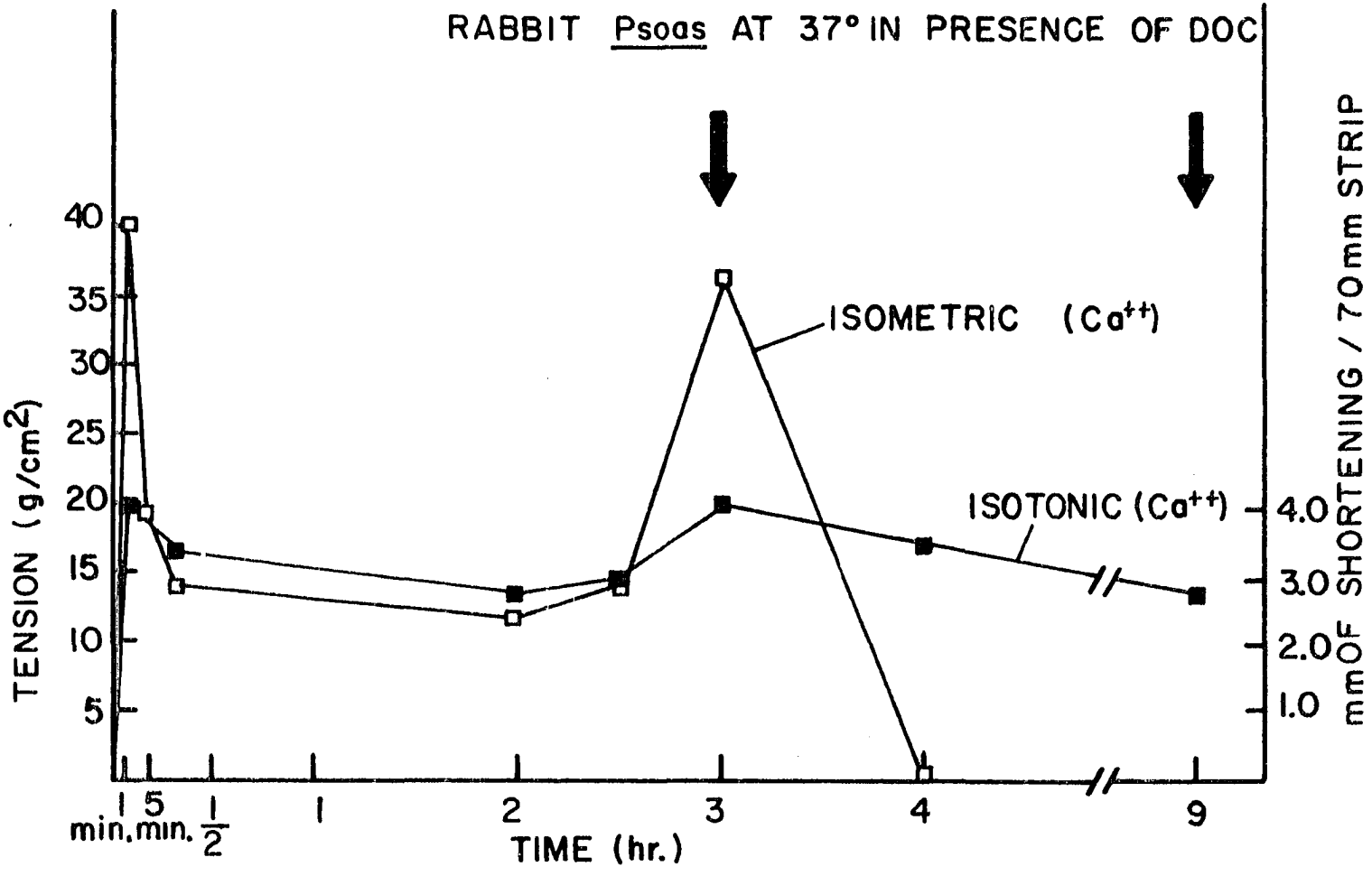
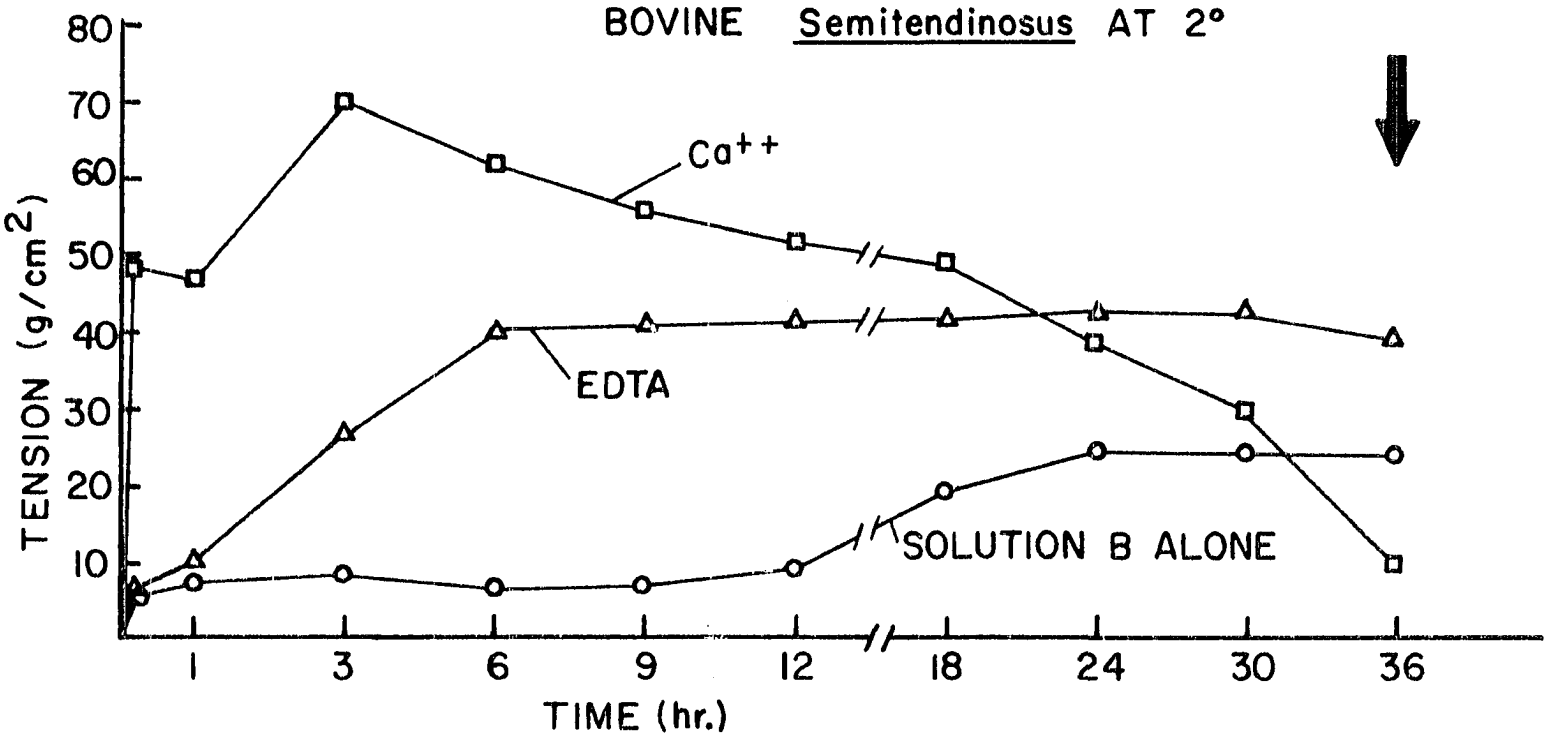

RABBIT Psoas AT 37° IN PRESENCE OF DOC
ISOMETRIC (Ca++)
ISOTONIC (Ca++)
TENSION (g/cm2)
40
35
30
25
20
15
10
5
4.0
3.0
2.0
1.0
mmOF SHORTENING / 70mm STRIP
1 min.
5 min.
1/2
1
2
3
4
9
TIME (hr.)

Figure 15. Effect of 1mM Ca^{++} and 1mM EDTA on post-mortem isometric tension development of bovine semitendinosus muscle at 2°. Bathing media were: solution B alone; solution B plus 1mM Ca^{++}; solution B (without $MgCl_2$) plus 1mM EDTA and 1mM deoxycholate. All measurements were made on one animal. Arrow indicates time at which a representative strip was removed for phase microscopy studies.


BOVINE Semitendinosus AT 2°
TENSION (g/cm²)
80
70
60
50
40
30
20
10
Ca++
EDTA
SOLUTION B ALONE
1
3
6
9
12
18
24
30
36
TIME (hr.)

times more tension than was developed by the strips in this experiment, and normally developed this tension earlier post-mortem.

The preceding studies, clearly indicated that Ca^{++} was potentiating a decline in post-mortem isometric tension development. Since it seemed probable that the rapid tension decline caused by Ca^{++} might be associated with structural changes in the myofibrils, a series of experiments were initiated to determine whether the presence of 1mM Ca^{++} in the saline bathing solution was causing any changes in myofibrillar ultrastructure. In these experiments, tension development of strips was monitored on the E and M physiograph, and at particular stages in tension development and decline, strips were removed from the myographs, homogenized in 0.25M sucrose, 0.05M Tris, 1mM EDTA, pH 7.6, and myofibrils prepared as described in Materials and Methods. These myofibrils were then examined with a phase-contrast microscope.

Immersion of muscle strips in a 1mM Ca^{++} solution caused a very drastic alteration in the structure of myofibrils prepared from these strips. The results shown in Plate 1 indicate that the presence of Ca^{++} in the bathing solution causes loss of Z-lines. This was most evident in rabbit psoas at 37° where the myofibril not only lost its Z-line, but was fragmented into myofibrils only 2-4 sarcomeres in length by the homogenization process (Plate 1d). This is in sharp contrast to those in solution B only (Plate 1a), or solution B-plus-deoxycholate (Plate 1b) where Z-lines are evident and myofibrils are not fragmented. In solutions containing Ca^{++}, addition of deoxycholate accelerated the rate of fragmentation, but the presence of deoxycholate was not a prerequisite for fragmentation (Plate 1e). In bovine semitendinosus at 2°, (Plate 1i), the presence of

Ca^{++} did not cause extensive fragmentation, but Z-lines were absent in most myofibrils examined. In rabbit psoas muscle at 2^{o}, small strips could be teased directly from the muscle strips taken from the physiograph. Direct examination of these strips in the phase microscope showed that many of them did not have Z-lines (Plate 1j). This indicates that Z-line removal occurs in the strip while it is attached to the myograph and is not necessarily a consequence of myofibril preparation.

Z-line removal is clearly due to Ca^{++} since myofibrils prepared from strips immersed in EDTA-containing solutions exhibited intact Z-lines and were not fragmented, and were usually 8-10 or more sarcomeres in length (Plate 1c, 1h). This was found even for myofibrils prepared from rabbit muscle strips that had been immersed in the EDTA-containing solution for 24 hours at 37^{o}. Furthermore, Z-line removal was not due to the presence of Mg^{++} (Mg^{++} was always omitted from the EDTA-containing solutions because EDTA chelates Mg^{++}) since myofibrils prepared from strips immersed in Mg^{++} and ImM EGTA (Plate 1g) exhibited a structure very similar to those stored in EDTA (Plate 1h).

The difference between EDTA-treated and Ca^{++} treated strips also was evident on a macromolecular scale. The gross structure of muscle strips treated with EDTA was well preserved, and very thin strips could easily be teased from this muscle. Moreover, these strips were not broken into individual myofibrils by teasing. On the other hand, muscle strips treated with Ca^{++} would break very easily during teasing and afterwards, short individual myofibrils could be found. This indicates that the lateral attachments of muscle fibers with one another were also weakened by treatment

Plate 1a. Phase micrograph of myofibril prepared from rabbit psoas after immersion in solution B for 24 hours at 37°. X2000.

Plate 1b. Phase micrograph of myofibril prepared from rabbit psoas after immersion in solution B plus ImM DOC for 24 hours at 37°. X2000.

Plate 1c. Phase micrograph of myofibril prepared from rabbit psoas after immersion in solution B (without $MgCl_2$) plus ImM DOC and ImM EDTA for 24 hours at 37°. X2000.

Plate 1d. Phase micrograph of myofibrils prepared from rabbit psoas after immersion in solution B plus ImM Ca^{++} and ImM DOC for 24 hours at 37°. Note the absence of Z-lines and the extreme fragmentation. X2000.

Plate 1e. Phase micrograph of myofibrils prepared from rabbit psoas after immersion in solution B plus ImM Ca^{++} for 24 hours at 37°. Note the absence of Z-lines and the fragmentation. X2000.

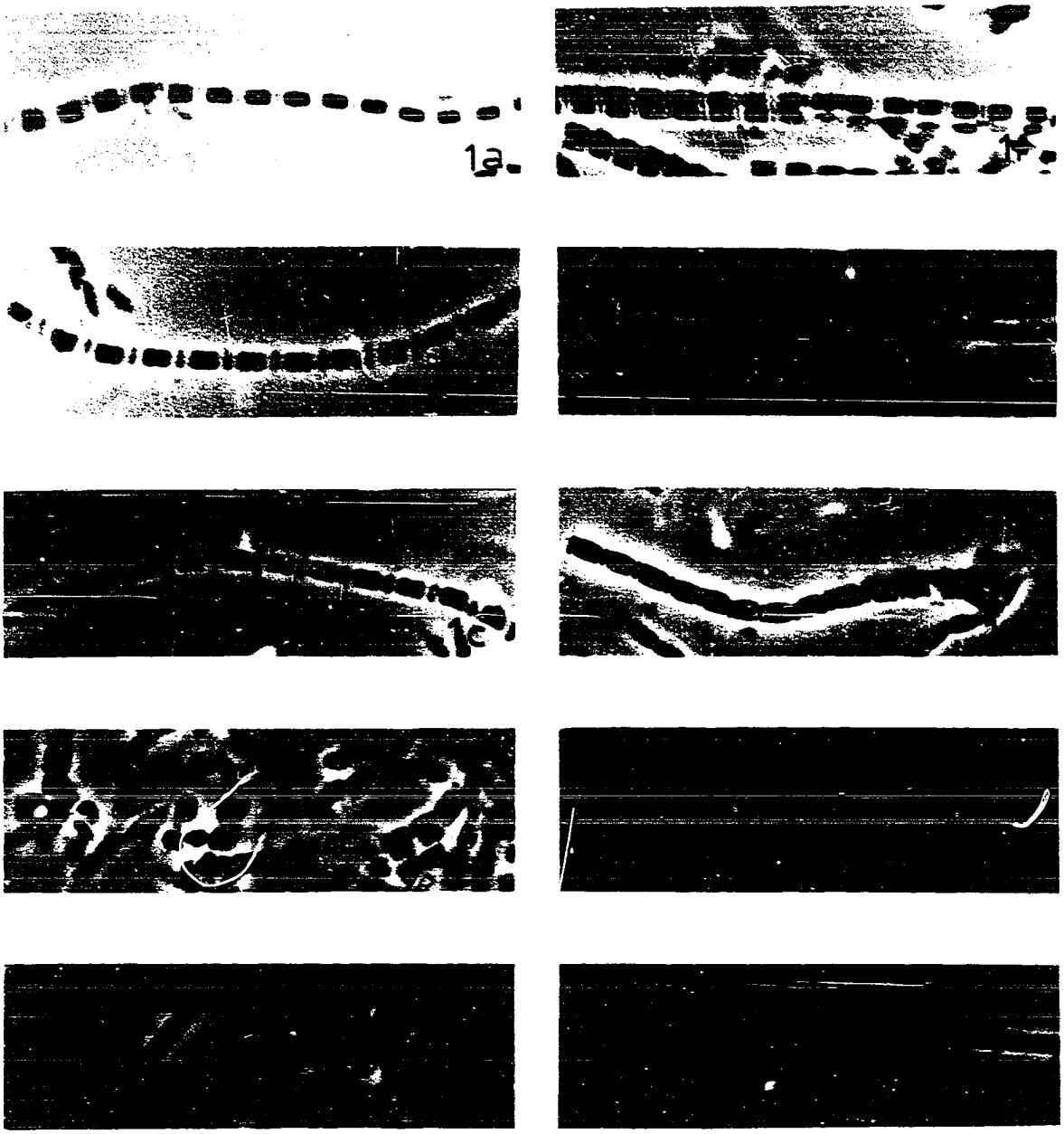
Plate 1f. Phase micrograph of myofibril prepared from bovine semitendinosus after immersion in solution B for 36 hours at 2°. X2000.

Plate 1g. Phase micrograph of myofibril prepared from bovine semitendinosus after immersion in Solution B plus ImM EGTA for 36 hours at 2°. Z-lines are more prominent in this myofibril than in the myofibril shown in Plate 1f. X2000.

Plate 1h. Phase micrograph of myofibril prepared from bovine semitendinosus after immersion in solution B plus ImM EDTA for 36 hours at 2°. Z-lines are more prominent in this myofibril than in the myofibril shown in Plate 1f. X2000.

Plate 1i. Phase micrograph of myofibril prepared from bovine semitendinosus after immersion in solution B plus ImM Ca^{++} for 36 hours at 2°. Note the absence of Z-lines. X2000.

Plate 1j. Phase micrograph of myofibril prepared from rabbit psoas after immersion in solution B plus ImM Ca^{++} and ImM DOC for 24 hours at 2°. Note the absence of Z-lines. X2000.


1a.

with Ca^{++}.

The phase microscopy studies clearly indicated that post-mortem storage of muscle strips in Ca^{++}-containing solutions caused removal of the Z-line. However, it was not possible with the resolution obtainable in the phase microscope to determine whether Ca^{++} caused a degradation of the thin filament which then resulted in loss of the Z-line, or whether the effect of Ca^{++} was more specifically on the Z-line itself. Consequently, an electron microscopy study was done in which strips were removed from the isometric myographs at particular points along the post-mortem tension pattern, fixed, embedded, sectioned, and examined in the electron microscope. Plate 2 shows the ultrastructure of a strip of rabbit psoas muscle fixed immediately after death. The structural features characteristic of uncontracted, striated muscle are evident. A wide H-zone is present in the center of the A-band, and the wide I-band is bisected by the Z-line. There is evidence of a five-period substructure in the M-line, which is located in the center of the H-zone. The Z-line has a fibrillar appearance and intact thick and thin filaments can be seen.

After suspension for three hours in solution B plus 1mM Ca^{++}, several marked changes have occurred in the ultrastructure of rabbit muscle (Plates 3a, 3b, 4a). Plate 3a shows the ultrastructure of a strip that had been suspended isotonically and loaded with just enough weight to keep the muscle taut in the bathing solution (cf. Figure 14). It is obvious that the sarcomeres of this strip have shortened extensively; I-bands have disappeared and it is difficult to detect either the M-line or Z-line.

Plate 2. Electron micrograph of rabbit psoas muscle sampled at death. All samples used for electron micrographs were fixed with gluteraldehyde and post-fixed with osmium tetroxide. Sections were stained with uranyl acetate and lead citrate. X41,731.

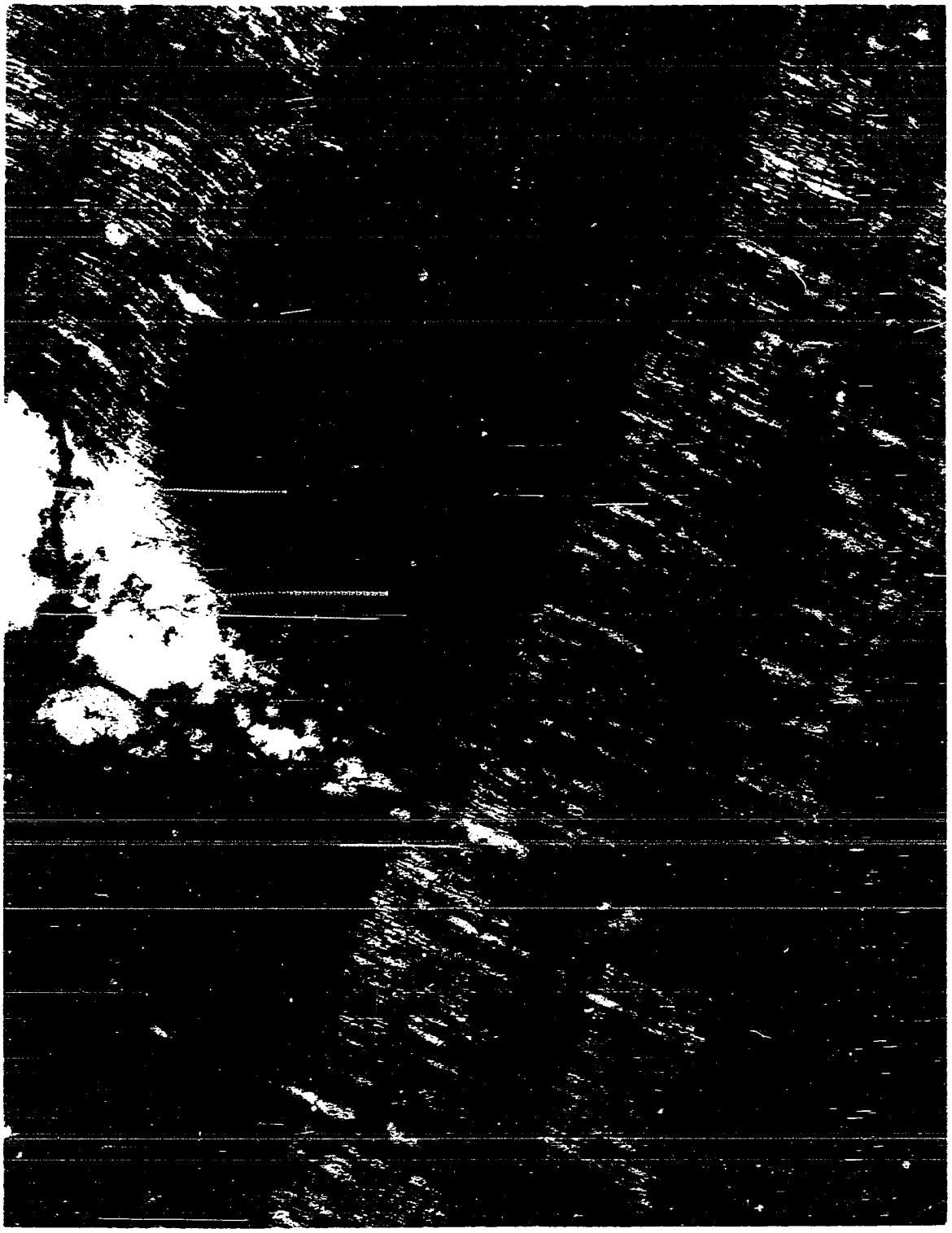
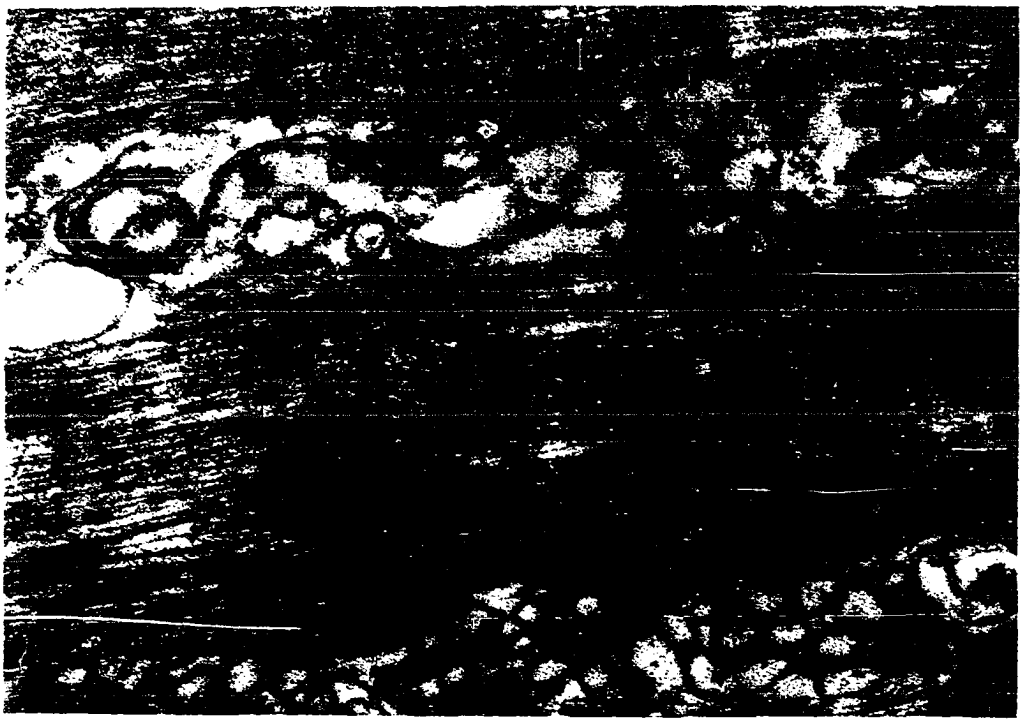
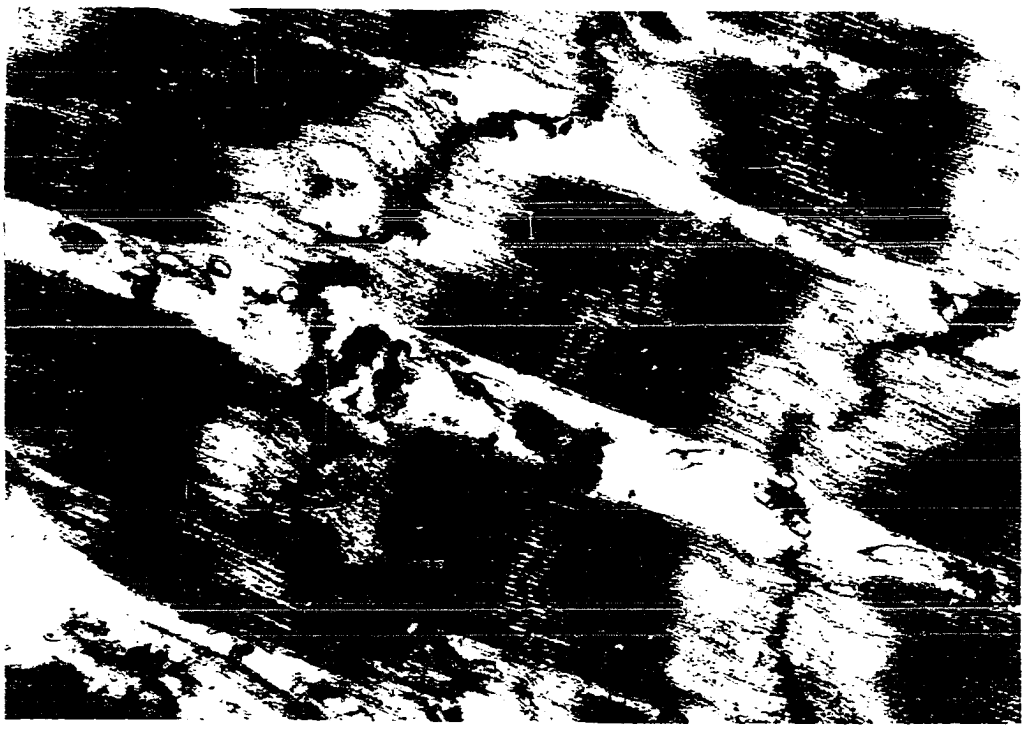


Plate 3a. Electron micrograph of rabbit psoas after being isotonically suspended for 3 hours in solution B plus ImM Ca^{++} and lmM DOC at 37°. X27,531. The supercontracted pattern usually seen at 3 hours with this treatment is evident, but characteristic ultrastructural features are difficult to distinguish.

Plate 3b. Electron micrograph of rabbit psoas after being isometrically suspended for 3 hours in solution B plus ImM Ca^{++} and ImM DOC at 37°. X27,531. Notice that the extensive shortening characteristic of isotonically suspended muscle does not occur in isometrically suspended muscle. Z-lines are present but show some discontinuities.





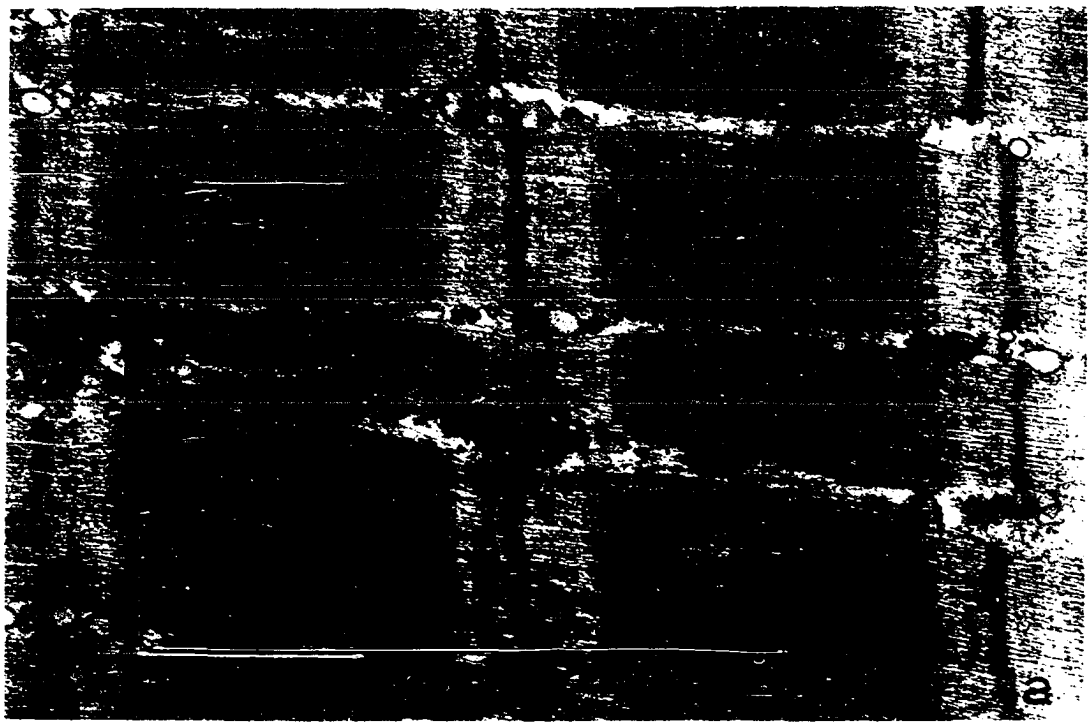
Under the conditions of this experiment (rabbit psoas at 37°, in solution B plus 1mM Ca^{++} and 1mM deoxycholate), maximum isometric tension (or isotonic shortening) has occurred after 3 hours (cf. Figure 14). Plates 3b and 4a show the ultrastructure of rabbit psoas muscle which had been suspended isometrically for 3 hours at 37° in solution B plus 1mM Ca^{++} and 1mM deoxycholate. As expected, isometric suspension prevented the severe shortening observed in the isotonically suspended fiber, but the sarcomeres in plate 3b and 4a have shortened from sarcomere lengths of 2.6μ at death to approximately 1.9μ at three hours. Some variation was encountered in the ultrastructure of isometrically suspended fibers after 3 hours in the presence of Ca^{++}. In some cases, the Z-line appeared intact and, except for slight shortening, the structure appeared very similar to the structure observed in at-death muscle (cf. Plate 2 and 4a). In other cases, the Z-line traced a tortuous path through the fiber, and breaks were evident in the Z-line (Plate 3b). In such fibers, the thin filaments were bent and exhibited a wavy appearance.

The ultrastructure of isometrically suspended rabbit psoas fibers after 3 hours in solution B plus 1mM EDTA and 1mM deoxycholate usually exhibited a severely contracted pattern. Under these conditions, the fiber appeared much like that shown for 3-hour, isotonically suspended Ca^{++}-treated fibers (Plate 3a). However, insufficient numbers of 3-hour, EDTA-treated fibers were examined to make a positive conclusion on the ultrastructure of these fibers. In all cases where such a judgement could be made, the Z-line of EDTA-treated fibers was intact after 3 hours in solution B.

The ultrastructure of isometrically suspended fibers after 9 hours in solution B plus 1mM Ca^{++} and 1mM deoxycholate is shown in Plates 4b and 5b.

Plate 4a. Electron micrograph of rabbit psoas after being isometrically suspended for 3 hours in solution B plus ImM Ca^{++} and ImM DOC at 37°. X29,597. Some variation is evident in muscle held isometrically for 3 hours. In this micrograph, fine structure including Z-lines, is better preserved than in Plate 3b.

Plate 4b. Ultrastructure of isometrically suspended rabbit psoas muscle after 9 hours in solution B plus ImM Ca^{++} and ImM DOC at 37°. X29,597. After 9 hours, Ca^{++} treatment has resulted in nearly complete removal of Z-line structure. Considerable shortening of sarcomeres has also resulted.



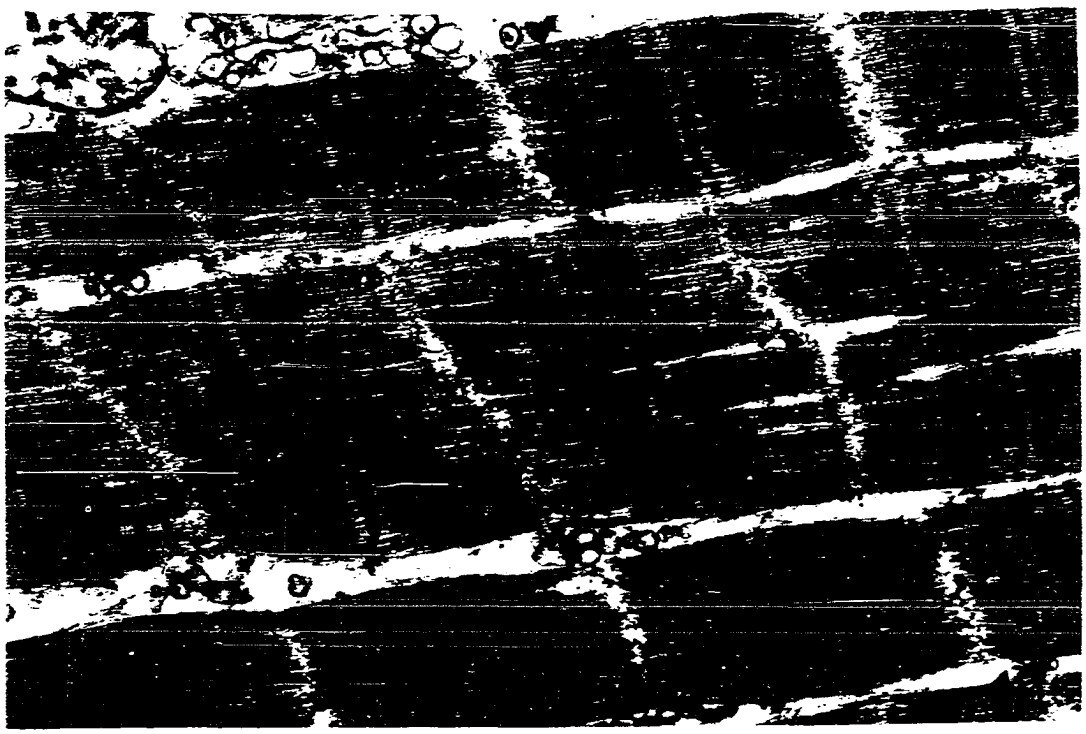


Plate 5a. Electron micrograph of isometrically suspended rabbit psoas muscle after 9 hours in EDTA at 37°. Note the prominent Z-lines and general state of structural preservation. The bathing solution contained solution B (without $MgCl_2$) plus ImM EDTA and ImM DOC. X27,531.

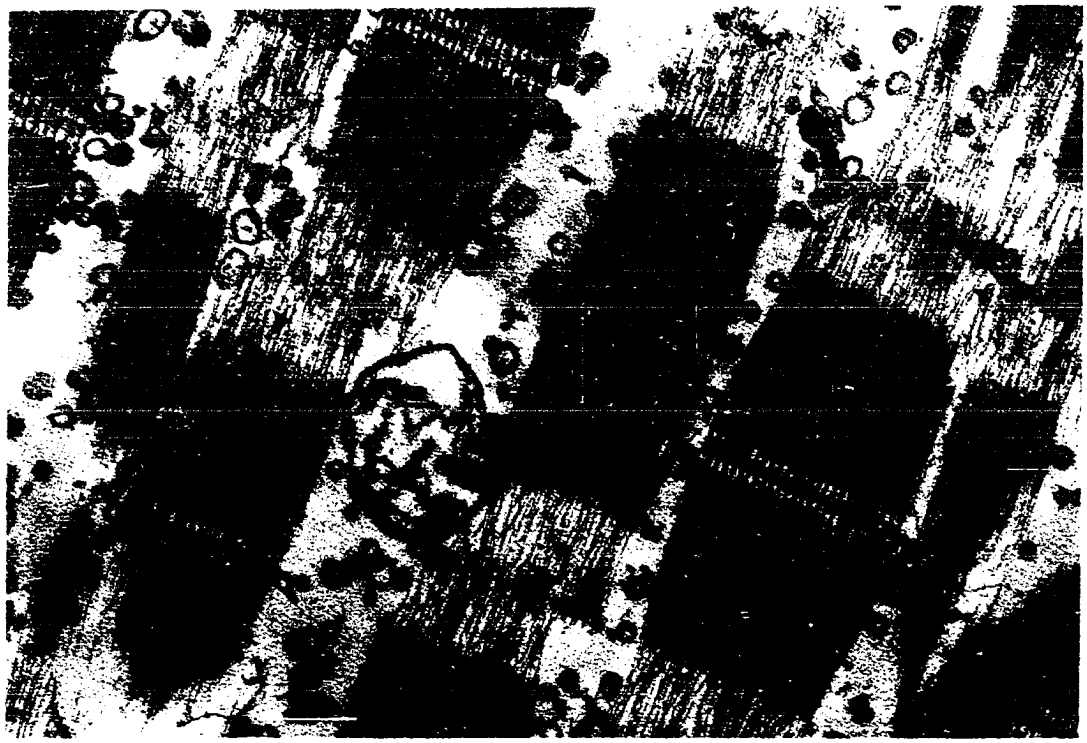
Plate 5b. Fine structure of rabbit psoas which was held isometrically for 9 hours at 37° in solution B plus ImM Ca^{++} and ImM DOC. In contrast to Plate 5a, Z-lines are not evident and sarcomeres are considerably shortened. X27,531.

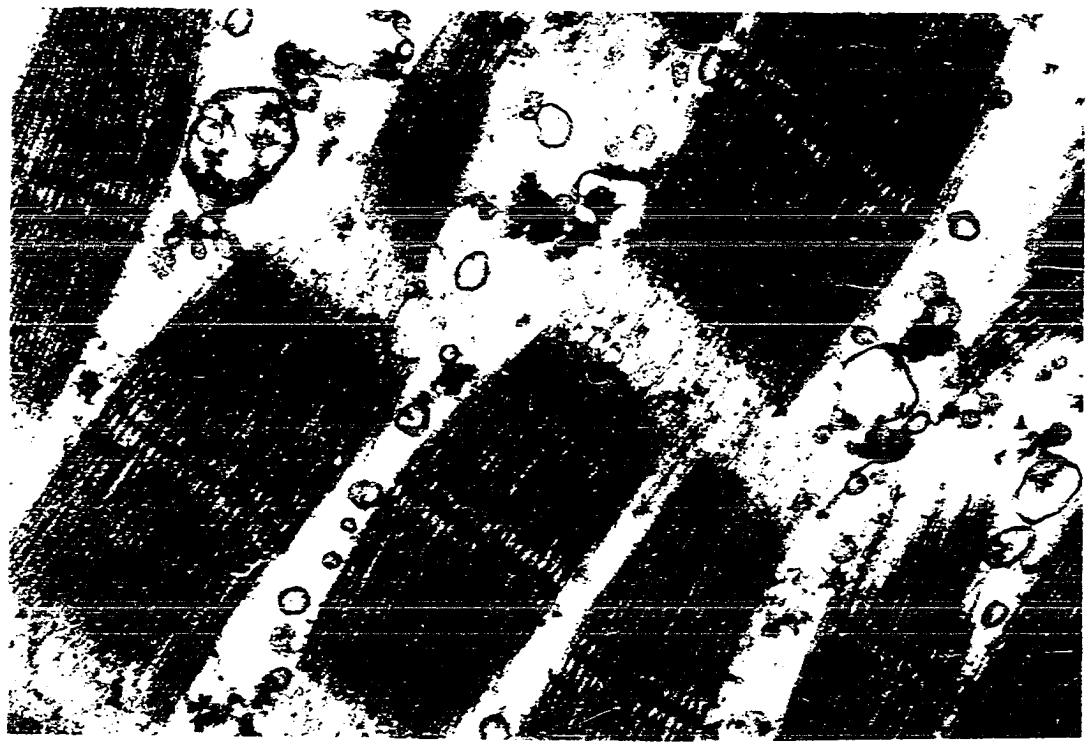
It is evident that almost complete removal of the Z-line has occurred between 3 and 9 hours post-mortem. There are still some small filaments extending across the area formerly occupied by the Z-line; these are more numerous in Plate 5b than in Plate 4b. The nature of these filaments is unknown. It is also obvious that the sarcomeres of isometrically suspended fibers have shortened to nearly 55% of rest length after 9 hours in solution B plus 1mM Ca^{++} and 1mM deoxycholate. Although it is surprising that sarcomeres in isometrically suspended muscle can undergo this degree of shortening, it is possible that this shortening occurs only after disintegration of the Z-line. The sarcomere would then be free to shorten without causing any change in length of the filaments. Because of the extensive shortening, it is difficult to ascertain the integrity of the thin filament in Plates 4b and 5b. However, a very clear thin filament overlap region is evident in the center of the A-bands in both plates, so thin filaments could not have undergone extensive degradation. It is also evident that M-lines are intact in Ca^{++}-treated muscle after 9 hours in solution B. Hence, the conditions in this experiment resulted in removal of Z-lines without affecting M-line structure. This is in contrast to the method used by Stromer et al. (1969) to extract Z-lines, since their method caused extraction of M-lines prior to extraction of Z-lines.

The structure of isotonically suspended muscle after 9 hours in solution B plus 1mM Ca^{++} and 1mM deoxycholate is shown in Plate 6a. The structure of this muscle is similar to the structure of isometrically suspended muscle subjected to the same conditions. However, in the

Plate 6a. Ultrastructure of isotonically suspended psoas muscle after 9 hours in solution B plus 1mM Ca^{++} and 1mM DOC at 37°. X26,371. Under these conditions, Z-lines are almost totally removed but the structure of the thin filaments is well preserved. Some shift in register of the filaments has occurred.

Plate 6b. Electron micrograph of rabbit psoas after being isometrically suspended for 24 hours in solution B plus 1mM Ca^{++} and 1mM DOC at 37°. X26,371. After 24 hours, treatment with Ca^{++} has resulted in total removal of Z-lines and small filaments extend through the area formerly occupied by the Z-line.





isotonically suspended muscle, the sarcomeres are not so severely shortened and intact thin filaments can be clearly seen. This suggests that the effect of Ca^{++} is on the Z-line itself, or on the I-Z junction, rather than on the thin filaments. The M-line is also intact in isotonically suspended muscle even though the Z-line is gone. Numerous filaments are evident in the area formerly occupied by the Z-line, and it appears that some overlap of thin filaments now occurs in this region (Plate 6a). Adjacent sarcomeres in the same fibril in Plate 6a appear to be shifted slightly out of register.

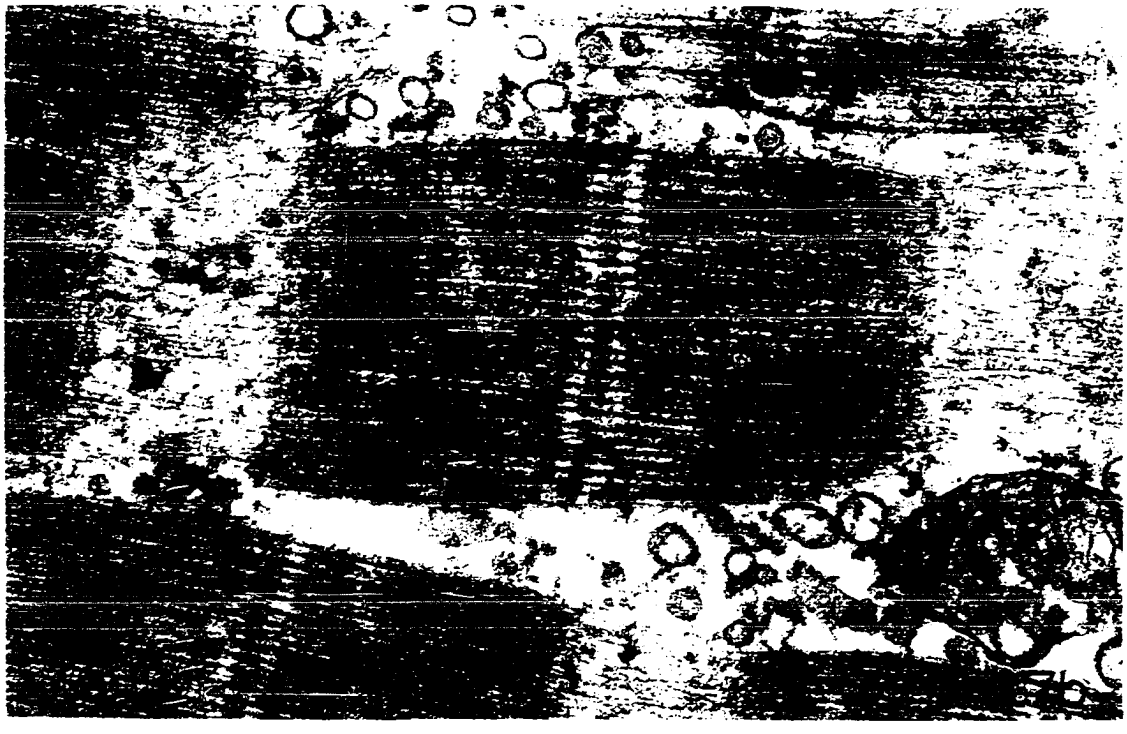
Plate 5a shows the ultrastructure of isometrically suspended rabbit psoas fibers after 9 hours in solution B plus 1mM EDTA and 1mM deoxycholate (without $MgCl_2$). Both the Z-lines and the M-lines are intact in this muscle, although the Z-line appears to have broadened slightly. Isometrically held fibers, even in the presence of extracellular EDTA have shortened slightly (average sarcomere lengths of 2.0μ compared to 2.6μ for at-death muscle). The A-I junction is somewhat more diffuse and less clearly delineated in the EDTA-treated fibers than in the at-death fibers (cf. Plates 2 and 5a). There are no other obvious structural differences between the 9-hour, EDTA-treated muscle and at-death muscle.

The ultrastructure of isometrically suspended rabbit psoas fibers 24 hours in solution B plus 1mM Ca^{++} and 1mM deoxycholate (Plates 6b and 7b) resembles that of the correspondingly treated fibers after 9 hours post-mortem. The M-lines are still intact in Ca^{++}-treated fibers after 24 hours post-mortem, and in fact, in Plate 7b, it is possible to see substructure in the M-lines of Ca^{++}-treated fibers. After 24 hours, the Ca^{++}-

Plate 7a. Electron micrograph of rabbit psoas muscle showing that after immersion in 1mM EDTA for 24 hours at 37°, Z-lines are very evident and have widened somewhat. Muscle strip was held isometrically in solution B (without Mg^{++}) plus 1mM EDTA and 1mM DOC. X41,539.

Plate 7b. Electron micrograph of rabbit psoas after being isometrically suspended for 24 hours in solution B plus 1mM Ca^{++} and 1mM DOC at 37°. X59,357. This higher magnification micrograph shows that after 24 hours of immersion in the Ca^{++} solution the Z-line structure appears totally absent, but M-line structure is well preserved. Small filaments are seen in the space previously occupied by the Z-line.


7a



treated fibers do not appear to be severely shortened as they were after 9 hours, and thin filaments now extend out past the edge of the A-band. It is therefore evident that the presence of 1mM extracellular Ca^{++} does not cause extensive degradation of thin filaments and that loss of the Z-line in Ca^{++}-treated fibers is due to an effect on the Z-line or the I-Z junction rather than an effect on the thin filaments. In addition, numerous filaments can be seen passing through the region formerly occupied by the Z-line in both Plates 6b and 7b.

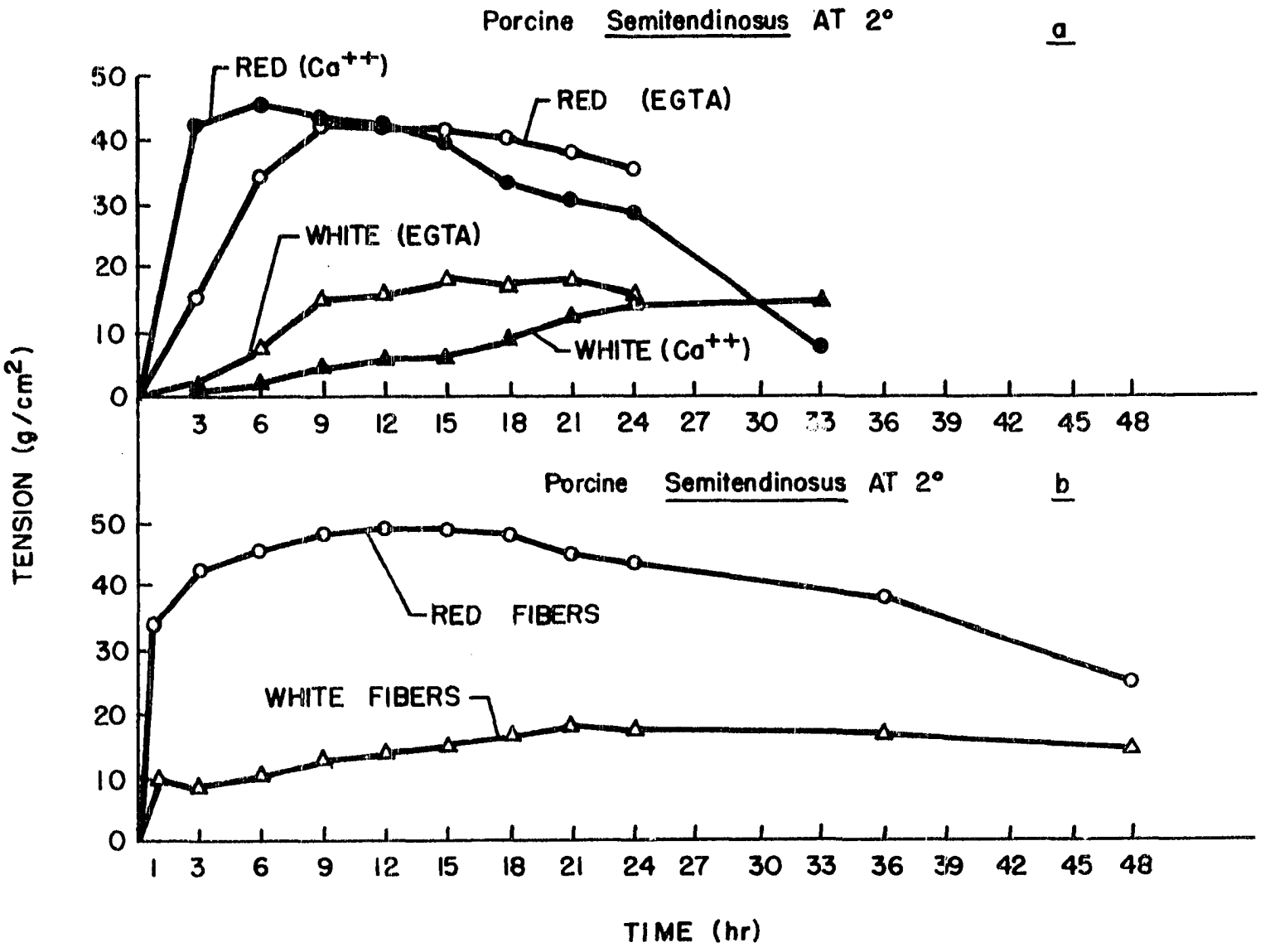
The ultrastructure of isometrically suspended rabbit psoas muscle after 24 hours in solution B plus 1mM EDTA and 1mM deoxycholate (without $MgCl_2$) is shown in Plate 7a. It is clear that EDTA-treated fibers retain both their Z-lines and their M-lines, even after 24 hours at 37°. The Z-line broadening noticed in 9-hour, EDTA-treated fibers is also evident in Z-lines of 24-hour, EDTA-treated fibers. This is in marked contrast to the in situ situation where Henderson (1968) found that Z-lines, and frequently M-lines also, were absent in rabbit muscle after 24 hours at 37°. Hence, it must be concluded that the presence of extracellular EDTA causes a remarkable preservation of M- and Z-line structure in post-mortem rabbit muscle. After 24 hours, sarcomeres of isometrically held, EDTA-treated fibers have lengthened. It is not clear whether some other mechanism is involved. Wide H-zones are not observed in Plate 7a; if relaxation had occurred by a sliding of interdigitating filaments, some increase in width of the H-zone would have been expected.

Studies on Red and White Muscle Fibers

It was shown earlier in this section that temperature dependence of post-mortem isometric tension development in bovine longissimus dorsi muscle was clearly different from the temperature dependence of isometric tension development in porcine or rabbit longissimus dorsi muscle (cf. Figures 3, 4, 5). It seemed possible that part of this difference may be due to the fact that bovine semitendinosus is a muscle containing predominantly red (Type I) fibers, whereas porcine and rabbit longissimus dorsi contain predominantly white (Type II) fibers. Consequently, a preliminary study was conducted to determine whether there was any clear difference between post-mortem isometric tension development of red and white muscles. The muscle chosen for this study was the porcine semitendinosus muscle. Beecher et al. (1965) have shown that this muscle is divided into two distinct parts, one consisting predominantly of red fibers, and the other consisting predominantly of white fibers. Furthermore, the white part of porcine semitendinosus contains as high a proportion of white fibers as any muscle in the porcine animal, and the red portion contains as high a proportion of red fibers as any muscle in the porcine animal. This presents a very advantageous situation since it is possible to obtain red and white muscle samples from areas located anatomically close to one another. The results of isometric tension experiments with the red and white semitendinosus muscle strips are shown in Figures 16a and 16b. In the presence of solution B alone (contaminating Ca^{++}), red fibers developed more tension and developed this tension sooner post-mortem than the white fibers (Figure 16b). Addition of either 1mM Ca^{++} or 1mM EGTA

Figure 16a. Effect of Ca^{++} and EGTA on post-mortem isometric tension development of muscle strips from the red and white portions of porcine semitendinosus muscle at 2°. Bathing media were: solution B plus 1mM Ca^{++}; solution B plus 1mM EGTA. Points are averages of measurements made on strips from two animals.

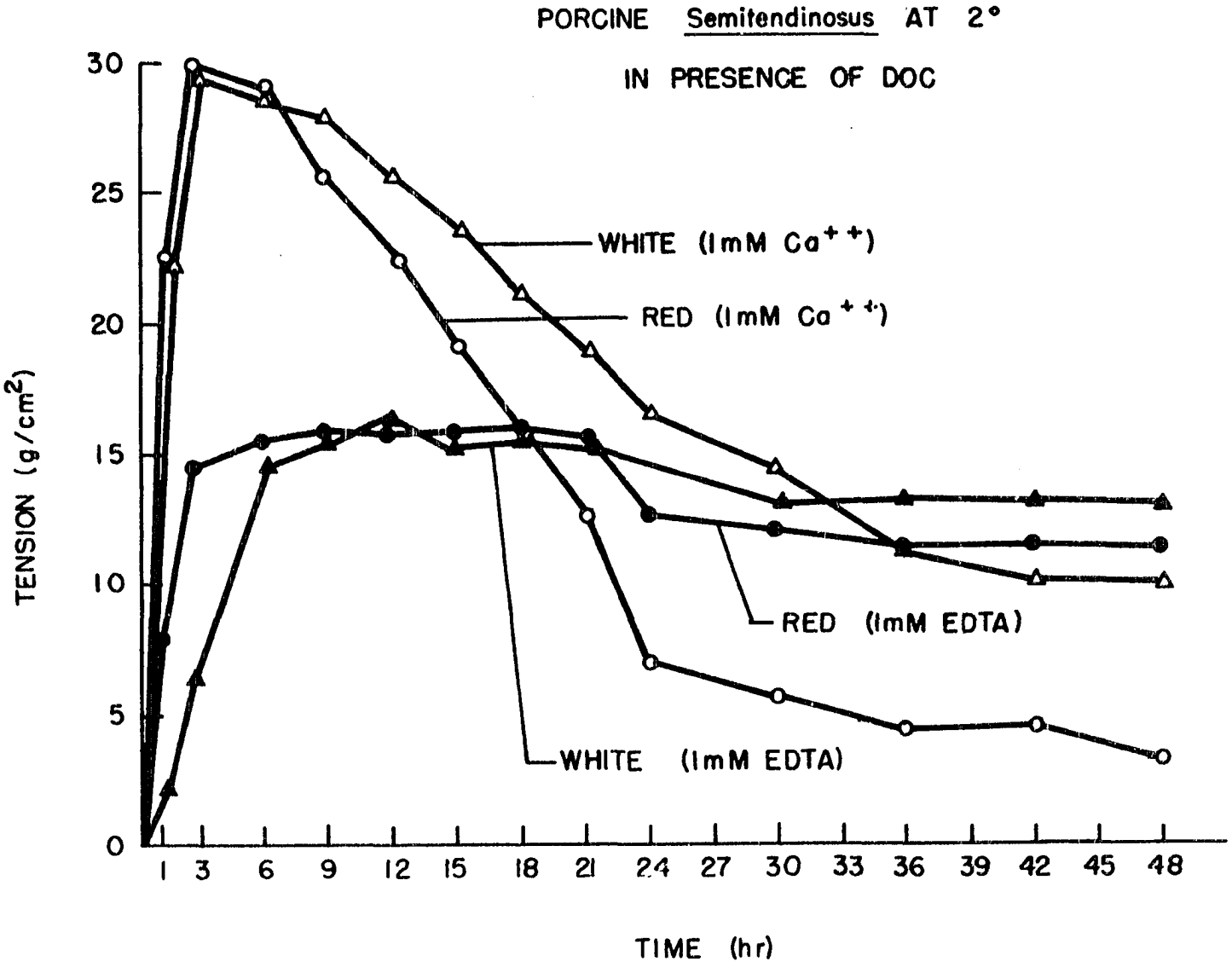
Figure 16b. Comparison of post-mortem isometric tension development of muscle strips from the red and white portions of porcine semitendinosus muscle at 2°. Bathing medium was solution B. Points are averages of measurements made on four strips from two animals.


Porcine Semitendinosus AT 2°
a
RED (Ca++)
RED (EGTA)
WHITE (EGTA)
WHITE (Ca++)
Porcine Semitendinosus AT 2°
b
RED FIBERS
WHITE FIBERS
TENSION (g/cm2)
TIME (hr)

to solution B did not cause any large change in tension development (Figure 16a), although addition of Ca^{++} to the red muscle strip resulted in a fairly rapid decline of isometric tension almost back to zero. Addition of 1mM deoxycholate to solution B caused a marked change in isometric tension development of red and white muscle strips (Figure 17). The presence of deoxycholate appears to eliminate much of the difference between isometric tension patterns of the two types of fibers. It is obvious that addition of deoxycholate caused a slight decrease in tension development of Ca^{++}-treated red muscle strips, and a marked increase in tension of Ca^{++}-treated white muscle strips (Figure 17). As was observed earlier, the presence of Ca^{++} causes isometric tension of both types of fibers to decline rapidly after maximum tension has been attained (about 1-3 hours post-mortem). In the presence of EDTA, on the other hand, isometric tension development is maintained at nearly a constant level out to 48 hours post-mortem.

These results suggest that the sarcolemmal membranes of red and white muscle fibers may differ significantly and that exposure to 1mM deoxycholate removes these differences. It has previously been reported that the sarcoplasmic and myofibrillar protein fractions and the sarcoplasmic reticulum fraction of red and white fibers differ. The difference in tension development between red and white porcine fibers at 2^{o} may also offer a partial explanation for the difference between results obtained in this study for tension development of porcine muscle at 2^{o}, and those obtained by Galloway and Goll (1967). Thus, the fact that Galloway and Goll found a cold shortening effect in porcine muscle at 2^{o} may be due to

Figure 17. Effect of deoxycholate on Ca^{++} or EDTA-stimulated isometric tension development of muscle strips from the red and white portions of porcine semitendinosus muscle at 2^{o}. Bathing media were: solution B plus 1mM Ca^{++} and 1mM deoxycholate; solution B (without $MgCl_2$) plus 1mM EDTA and 1mM deoxycholate. Points are averages of measurements made on four strips from two animals.


PORCINE Semitendinosus AT 2°
IN PRESENCE OF DOC
TENSION (g/cm²)
TIME (hr)
WHITE (1mM Ca++)
RED (1mM Ca++)
RED (1mM EDTA)
WHITE (1mM EDTA)
0
5
10
15
20
25
30
1 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48

the circumstance that their strips contained a higher proportion of red fibers than were contained by the strips used in this study.

DISCUSSION

The results of this investigation have several important implications in both muscle biology and the mechanism underlying development and resolution of rigor mortis. Several problems inherent in using intact muscle fibers to study post-mortem changes in muscle are also plainly evident from this investigation. As will be pointed out, it was not possible to circumvent all these problems, but the results clearly indicate some important relationships among post-rigor softening, loss of Z-line structure, and Ca^{++}.

Almost all findings of this investigation were made possible by the discovery that post-mortem isometric tension patterns of muscle strips are nearly identical whether the strips are incubated in humid air or in a saline solution. However, use of a saline solution in post-mortem isometric tension measurements has several distinct and important advantages over use of humid air: 1) incubation of strips in saline solution prevents the dehydration often encountered with strips suspended in air; 2) incubation in saline solution increases the precision of temperature regulation in those experiments investigating the effect of temperature on isometric tension; moreover, by use of pre-cooled solutions, it is possible to lower the temperature of muscle strips much more quickly than was possible in air; 3) bacteriostatic or bacteriocidal agents such as NaN_3 can conveniently be included in the saline solutions, thereby eliminating bacterial action as a cause of post-mortem tension development and decline; 4) incubation of muscle strips in saline solutions makes it possible to

easily and conveniently determine the effect of changing external pH, ionic strength, or chemical environment on post-mortem isometric tension patterns. This latter advantage was particularly important during the course of this investigation.

A second important methodological contribution to this investigation was the availability of an E and M physiograph plus five isometric myograph transducers to measure isometric tension. This apparatus made it possible to measure post-mortem isometric tension on five strips from a single animal. Since animal variation is one of the largest sources of variation in post-mortem isometric tension measurements, the simultaneous use of five isometric myograph transducers not only increased by fivefold the number of isometric tension measurements it was possible to do, but also eliminated an important source of variation and thereby increased the precision of the experiments. Hence, many experimental results could be checked by testing the effects of all treatments on strips from a single animal. This was an important advantage over the earlier "isometer" instrument (Busch et al., 1967; Jungk et al., 1967) which measured isometric tension on only one strip at a time. Moreover, it became evident during the course of this investigation that the E and M physiograph and isometric transducers were more sensitive to small changes in isometric tension development than the original "isometer"; this increased sensitivity resulted in some changes in existing concepts concerning the effect of temperature on post-mortem isometric tension patterns.

With this improved experimental setup, it was possible to clearly demonstrate that both development and decline of isometric tension occurs

in post-mortem rabbit, bovine, and porcine muscle at all temperatures between 2° and 37°. Previous results had indicated that rabbit muscle did not develop post-mortem isometric tension at 2°. This now appears to be due to the lower sensitivity of the original isometer. Moreover, it had previously been difficult to demonstrate a decline in post-mortem isometric tension at temperatures above 10°, and it had been suggested that loss of ability to maintain isometric tension occurred only in bovine muscle at 2° (Busch et al., 1967). It is now evident, however, that these results were due to dehydration of the muscle strips at higher temperatures and that loss of ability to maintain isometric tension does in fact occur in bovine, porcine, and rabbit muscle at all temperatures between 2° and 37°. Moreover, the results of this study suggest that isometric tension decline is faster and more dramatic when tension development is greater. The reason for this will be discussed later.

Availability of the E and M physiograph also made it possible to measure post-mortem isometric tension development, isotonic shortening, and loss of extensibility simultaneously in a series of muscle strips from the same animal. Such experiments have shown that isotonic shortening and isometric tension development of post-mortem muscle strips closely parallel one another. However, changes in length of isotonically suspended fibers are much smaller than the corresponding changes in isometric tension. Moreover, although loss in ability to maintain isometric tension is paralleled by lengthening of isotonically suspended fibers, this lengthening occurs very slowly and to a much lesser extent than the isometric tension

decline. In spite of these differences in magnitude, it is clearly possible to detect a resolution of rigor in both isometrically and isotonically suspended fibers. However, the isometrically suspended fibers clearly constitute the most sensitive system for detecting small changes during the onset or resolution of rigor mortis. These findings support Goll's (1968) contention that there is a resolution of rigor mortis.

The extensibility measurements, on the other hand, do not closely parallel the isometric tension measurements. Loss of extensibility does not begin until maximum isometric tension has almost been reached. This may reflect a difference in sensitivity of the methods used to detect extensibility changes and isometric tension development, or it may indicate that the extensibility measurement is not a sensitive indicator of changes in post-mortem muscle. In confirmation of earlier results (Bate-Smith and Bendall, 1949), this study found that once post-mortem muscle loses its extensibility, it does not regain it again. Thus, the extensibility measurement does not undergo any change corresponding to the isometric tension decline. Since the isometric tension method should expose a muscle strip to conditions very similar to those experienced by a muscle left attached to the carcass, and since the isometric tension technique appears to be a more sensitive indicator of post-mortem changes in muscle, it is clear that isometric tension constitutes the best single physical measurement of post-mortem events in muscle.

Measurements made with the improved instrumentation used in this investigation have changed some existing concepts concerning the rate and extent of post-mortem tension development at different temperatures. Thus,

maximum post-mortem tension in porcine, bovine and rabbit muscle is reached soonest post-mortem at 37°, although tension development in the bovine begins sooner post-mortem at 2° than at 37°. Also, the effect of temperature on isometric tension development and decline is very similar in both rabbit and porcine muscle, with tension development in both species being maximum at 37° and very similar at temperatures between 2° and 25°. However, temperatures of 25° or above, cause greater tension development in rabbit muscle than in porcine muscle. Post-mortem isometric tension development of bovine muscle, on the other hand, exhibits an entirely different temperature dependence than post-mortem isometric tension of porcine or rabbit muscle. Hence, it was shown earlier that post-mortem isometric tension development was maximal at 2° in bovine muscle (Busch et al., 1967; Locker and Hagyard, 1963), but it was not clear before now that bovine muscle at 37° develops much less isometric tension than porcine or rabbit muscle at 37°. The reasons for these differences between bovine, porcine and rabbit muscle are not clear. It may be related in part to the finding in this study that red fibers develop greater isometric tension development at 2° than white fibers. It is clear that bovine muscle possesses a greater proportion of red fibers than porcine or rabbit muscle. A higher proportion of red fibers in Galloway and Goll's (1967) samples may also account for their report that porcine muscle develops more isometric tension at 2° than at 37°. It is evident from the present investigation, however, that porcine longissimus dorsi muscle (primarily white fibers) develops about three times more post-mortem isometric tension at

37° than it does at 2°.

The isometric tension results of this study do support Henderson's (1968) microscopic evidence on the effect of temperature on post-mortem shortening in rabbit, porcine and bovine muscle. Thus, on the basis of sarcomere length measurements made on both myofibrils and sectioned muscle, Henderson (1968) concluded that maximum shortening of unrestrained muscle from all three species occurred soonest post-mortem at 37°. Also, Henderson's study indicated that unrestrained porcine and rabbit muscle exhibited a very similar post-mortem shortening pattern at all temperatures between 2° and 37°. Unrestrained bovine muscle underwent the greatest degree of post-mortem shortening at 2° whereas unrestrained rabbit and porcine muscle experienced the greatest degree of post-mortem shortening at 37°. Consequently, there is close agreement between the isometric tension results of this study and the degree of shortening observed by Henderson (1968) in unrestrained muscle. However, as pointed out by Henderson, the amount of shortening of unrestrained muscle is not necessarily directly related to amount of isometric tension development.

The dependence of post-mortem isometric tension development on temperature may be related to post-mortem changes in meat tenderness. Busch et al. (1967) and Marsh and Leet (1966) have previously shown that post-mortem shortening of bovine muscle at 2° causes decreased tenderness if this shortening does not exceed 40% of the muscle's rest length. The fact that neither porcine nor rabbit muscle shortens extensively at 2° may account, in part, for the greater tenderness of meat from these two species.

However, in normal meat handling procedures, muscles are not excised from the carcass immediately after death, and therefore, the practical importance of muscle shortening to meat tenderness is presently not clear.

The results of this study clearly show that post-mortem bovine, porcine, and rabbit muscle exhibit both a development and a decline of isometric tension. However, efforts to isolate the factors responsible for this tension development and decline have been hampered by difficulties in ascertaining whether the sarcolemmal membrane was permeable to substances added to the saline solution bathing the fiber. It was possible to show that varying pH of the bathing solution from 5.0 to 7.0 did not eliminate post-mortem isometric tension development and decline of rabbit psoas fibers at either 2^{o} or 37^{o}. However, there have been several reports (Rome, 1968; April et al. 1968) indicating that intracellular pH of muscle fibers is not greatly affected by changes in extracellular pH, and that variations in extracellular pH affect principally the selective permeability of the sarcolemma, making it more or less permeable to certain ions. Hence, the fact that decreasing extracellular pH from 7.0 to 5.0 shortens the time required for rabbit psoas to develop isometric tension at 37^{o} may be explained by a greater sarcolemmal permeability to KCl at lower pH values. This increased permeability would result in a decrease in intracellular KCl concentration which in turn would cause tension development sooner post-mortem (April et al. 1968). If this is the reason for the early isometric tension development at pH 5.0, lowering the temperature to 2^{o} must have just the opposite effect on sarcolemmal permeability, because

tension development at 2^{o} occurs sooner post-mortem at higher pH values. Regardless of the cause for these pH effects on post-mortem isometric tension patterns, it is clear that variations in extracellular pH per se are not the cause of post-mortem isometric tension development and decline. Moreover, since the hydrogen ion has a very small radius, it is reasonable to assume that the large extracellular pH differences used in this study also caused some changes in intracellular pH, and therefore that variations in intracellular pH are also not a causative factor in post-mortem tension development and decline. This conclusion is supported by the finding that storage of isolated myofibrils for periods up to 7 days at pH values ranging from 5.5 to 8.0 did not change the Ca^{++}-modified, the Mg^{++}-modified, nor the Mg^{++}-EGTA-modified ATPase activities of the myofibrils. Together, these results provide substantial evidence that intracellular pH variations are not a causative factor in post-mortem muscle tension development and decline.

The attempts to determine whether proteolytic enzymes were involved in post-mortem isometric tension decline also were impeded by inability to ascertain the state of the sarcolemmal membrane permeability. Since trypsin can quickly and efficaciously remove Z-lines from myofibrils, the presence of trypsin should prevent post-mortem isometric tension development altogether, or at least cause a very rapid decline in tension development. The fact that trypsin's presence in the bathing medium had no effect on post-mortem isometric tension patterns, even in the presence of deoxycholate, almost certainly indicates that trypsin was not able to reach the myofibrils. Consequently, the results of this study failed to provide

any clear evidence on the role of catheptic enzymes in post-mortem isometric tension decline. However, since trypsin seemed unable to penetrate the sarcolemmal membrane, even after 8-9 hours at 37°, in the presence of 1mM deoxycholate, these results do clearly show that any catheptic proteolysis of muscle must originate from cathepsins present inside the cell at death, and not from cathepsins present in leucocytes located in the capillaries outside the muscle cell. Hence, although this study fails to provide any definitive evidence on the role of proteolysis in meat tenderness, it does add further to the already weighty evidence that proteolysis is not an important factor in meat tenderness.

The most interesting results of this investigation concern the effect of Ca^{++} on post-mortem isometric tension development and decline. Greaser et al. (1967) and Eason (1969) have shown that during the first 2-3 hours after death, sarcoplasmic reticular membranes rapidly lose the ability to sequester Ca^{++}. Since over 70% of the muscle cell's Ca^{++} is sequestered by sarcoplasmic reticular membranes, loss of the Ca^{++}-sequestering ability of these membranes results in release of this bound Ca^{++}. This release probably initiates post-mortem isometric tension development. Consequently, it was surprising to discover that addition of Ca^{++} to the saline solution bathing the muscle strips did not cause immediate development of post-mortem tension, and even more surprising to learn that addition of EDTA to the bathing medium did not inhibit post-mortem tension development but actually appeared to potentiate it. Since EDTA chelates both Mg^{++} and Ca^{++}, and both of these cations are obligatory requirements for muscle contraction, it is almost certain that the added

EDTA is not penetrating the sarcolemmal membrane during the first 4-6 hours post-mortem, when the EDTA-potentiated shortening occurs. Moreover, intracellular Ca^{++} and Mg^{++} must also be unable to pass through the sarcolemmal membrane, since if they could, these cations should respond to the concentration gradient and diffuse gradually out of the cell and there be chelated by the EDTA. This process should also totally prevent any post-mortem isometric tension development. This reasoning further suggests that any Ca^{++} added to the bathing medium is also unable to pass through the sarcolemma, since it seems likely that if intracellular Ca^{++} is unable to diffuse out, then extracellular Ca^{++} is also probably unable to diffuse in. This would account for failure of added extracellular Ca^{++} to cause immediate isometric tension development. Addition of 1mM deoxycholate in this study did not appear to enhance the permeability of the sarcolemmal membrane to either EDTA or Ca^{++}. Although the sarcolemma appears impermeable to either added Ca^{++} or EDTA, it is nevertheless clear that addition of these substances to the bathing medium does affect post-mortem isometric development. EDTA appears to actually potentiate tension development. The reason for this effect is presently not known, but it presumably originates from an effect of EDTA on the sarcolemmal membrane.

The available evidence already suggests that EDTA and Ca^{++} cannot penetrate the sarcolemma during the first 5-6 hours post-mortem, when isometric tension development is occurring, but there are several indications that both extracellular Ca^{++} and EDTA can exert some influence directly on the myofibrils after 5-6 hours post-mortem (after maximum tension development). For example it was observed that, in the presence of 1mM added

deoxycholate and Ca^{++}, isometric tension decline was very rapid and dramatic, and tension development quickly returned to zero. In the presence of 1mM added EDTA, however, isometric tension decline was imperceptible, and tension development remained constant for 24 hours. Rate of isometric tension decline in the absence of either added Ca^{++} or added EDTA was intermediate, since such solutions contain approximately $5x10^{-6}M$ contaminating Ca^{++}, and since the sarcoplasmic reticulum of the muscle cell also releases some Ca^{++} during the first several hours post-mortem. Consequently, it is necessary to add a Ca^{++} chelator, such as EDTA, to lower Ca^{++} concentration to the point that isometric tension does not decline. Since EDTA will bind Mg^{++} as well as Ca^{++} and since solution B did not contain $MgCl_2$ when EDTA was to be added, it might be argued that the preservation of post-mortem isometric tension development was due to removal of Mg^{++} rather than Ca^{++}. However Ca^{++} removal was implicated in the tension preservation effect by measuring tension development in the presence of added EGTA, a chelator which binds Ca^{++} but not Mg^{++}. In this experiment, solution B contained 5mM $MgCl_2$. Addition of 1mM EGTA was shown to cause preservation of post-mortem isometric tension in a manner similar to that observed for EDTA.

Goll (1968) has suggested that loss of the ability to maintain isometric tension development in post-mortem muscle is due to a weakening of the actin-myosin interaction, this weakening causing some "slippage" at the points where myosin cross-bridges interact with actin. Since this "slippage" should result in longer sarcomeres, several experiments were done in which muscle strips were removed from the isometric myograph

transducer at either the point of maximum tension or at the time when tension of the Ca^{++}-treated fibers had returned to zero. Myofibrils were prepared from these strips and examined in the phase microscope. This study showed that added extracellular Ca^{++} causes removal of Z-lines from myofibrils. Myofibrils prepared from Ca^{++}-treated fibers after 9 or 24 hours post-mortem at 37° had no Z-lines and were highly fragmented, often being only two or three sarcomeres in length. On the other hand, myofibrils prepared from EDTA-treated fibers after 9 to 24 hours post-mortem at 37° exhibited intact Z-lines and were usually 8-10 sarcomeres in length. This preservation of myofibril structure was also observed when EGTA was added to the bathing medium, thereby demonstrating that Z-line removal was in fact due to Ca^{++} and not to Mg^{++}. The fragmentation of myofibrils prepared from post-mortem muscle has been observed previously (Davey and Gilbert, 1969; Henderson, 1968; Stromer and Goll, 1967a; Takahashi et al., 1967) and is generally attributed to weakening at the Z-line. The preservation effect of added EDTA or EGTA on myofibrillar structure is quite remarkable since Henderson (1968) has shown that myofibrils prepared from rabbit muscle stored in air for 4 hours at 37° are fragmented and have no Z-lines. The loss of Z-lines in muscle stored in air must be attributed to release of Ca^{++} from the denatured sarcoplasmic reticular membranes. This release of intracellular Ca^{++} probably causes a muscle Ca^{++} concentration of 10^{-4}M. Consequently, the addition of EDTA extended the time required for Z-line removal from post-mortem muscle from 4 hours to past 24 hours post-mortem.

Electron micrographs of sectioned samples from the isometrically or

isotonically suspended muscle strips confirmed the conclusion that added Ca^{++} causes Z-line removal from such strips and demonstrated that absence of Z-lines in myofibrils examined in the phase microscope was not due to Z-line removal by the myofibril preparation procedure. The electron micrographs further showed that the effect of Ca^{++} is on the Z-line itself since after 9 hours post-mortem, the Ca^{++}-treated myofibrils had no Z-lines but did exhibit intact thin filaments. Furthermore, several instances were seen where the Z-line had apparently split down its center and one half was left attached to each of opposite sarcomeres. Thus, the effect of Ca^{++} seems to be on the Z-line itself rather than a breakdown of thin filaments, resulting in a release of the Z-line. The Ca^{++}-induced degradation of the Z-line appears to occur most rapidly at 37^{o} in the presence of deoxycholate, but it was shown to also occur at 2^{o} and in the absence of deoxycholate. Since deoxycholate by itself does not appear to have any effect on the Z-lines of myofibrils, the role of deoxycholate is apparently to assist in making the sarcolemma permeable to the added Ca^{++}, thereby increasing the availability of the added Ca^{++} to the myofibrils. Deoxycholate may also exert a small additional effect by aiding in maximal release of Ca^{++} sequestered by the sarcoplasmic reticulum.

The preceding evidence clearly links Ca^{++} to Z-line degradation in post-mortem muscle fibers, and furthermore, suggests that Z-line degradation is directly related to post-mortem isometric tension decline. Goll (1968) indicated that Z-line degradation was probably responsible for the increased fragility of post-mortem muscle fibers, but suggested that loss

of ability to maintain isometric tension was likely due to weakening of the actin-myosin interaction. The results of the present study do not provide any evidence concerning the role of the actin-myosin interaction in post-mortem isometric tension decline, but they do provide clear evidence for a direct relationship between loss of Z-lines and isometric tension decline in post-mortem muscle. Thus, whenever isometric tension decline occurred rapidly and completely, it could be shown that extensive degradation of Z-lines had also occurred. Conversely, when isometric tension decline of post-mortem muscle strips was imperceptible, Z-line structure of these strips appeared intact. Consequently, it now appears that loss of Z-lines may be the principal reason for post-mortem isometric tension decline.

Since Ca^{++} is clearly implicated in Z-line removal in post-mortem muscle, it was of interest to determine the effects of Ca^{++} on isolated myofibrils. Myofibrils were prepared from at-death muscle and incubated at 2^{o} in 150mM KCl, 0.05 M Tris-acetate, pH 7.0, and 1mM Ca^{++} for periods up to 7 days. The added Ca^{++} had no visible effect on the Z-line in these myofibrils. Moreover, Z-lines of the Ca^{++}-treated myofibrils did not appear any more fragile to shear forces created by homogenization than Z-lines from untreated myofibrils. Consequently, the exact nature of the Ca^{++} effect on post-mortem muscle remains unknown. In spite of the large amount of indirect evidence against involvement of catheptic proteolysis in post-mortem myofibril disintegration, it is nevertheless possible that Ca^{++} induced Z-line removal in post-mortem fibers is due to a Ca^{++}-activated protease. This protease would obviously be absent in the myofibril

preparations.

It is also possible that Ca^{++} acts on Z-lines only during isometric tension development. This hypothesis presumes that during tension development, the nature of the Z-line or the I-Z junction changes and certain Ca^{++}-labile bonds are exposed. Thus, loss of the sarcoplasmic reticulum's ability to sequester Ca^{++} may cause both shortening and loss of Z-line structure in post-mortem muscle. A very rapid release of Ca^{++} from the denatured sarcoplasmic reticular membranes would cause a rapid development of tension, and because of the simultaneous effect of Ca^{++} on the Z-line, would also cause a rapid release of isometric tension. Hence, in terms of this second hypothesis, rate of tension decline should be most rapid when the rate of tension development is fastest. It is interesting to note that Henderson (1968) found that Z-line degradation in bovine, procine, and rabbit muscle was most extensive at 37^{o}, where unrestrained shortening of the muscles also occurred soonest post-mortem. Furthermore, it was shown in the present study that isometric tension decline is fastest when rate of tension development is fastest. Therefore, the findings of the present study indicate that release of Ca^{++} from the sarcoplasmic reticular membranes causes both development and decline of isometric tension in post-mortem muscle. The release of Ca^{++} causes initiation of shortening at once, but Ca^{++} can cause Z-line degradation only after tension development has altered the I-Z junction, making Ca^{++}-labile bonds available to the Ca^{++}. This explains why tension decline does not occur until several hours after tension development.

SUMMARY

The isometric tension development of post-mortem rabbit, porcine, and bovine muscle strips has been studied at 2, 16, 25, and 37° and under a variety of conditions. Muscle strips were attached at one end to a glass rod projecting from the side of an 800 ml. beaker. The other end was attached to an isometric myograph transducer. Tension was recorded by use of a six-channel E and M physiograph. It was shown that isometric tension patterns of post-mortem muscle strips were identical whether the strips were suspended in a dilute saline solution or in humid air. However, isometric tension measurement in solution has several advantages over isometric tension measurement in air: 1) suspension of strips in solution prevents dehydration which occurs when strips are suspended in humid air, particularly at temperatures above 10°; 2) suspension of strips in solution makes it possible to obtain more rapid temperature equilibration and more precise temperature control during investigations on the effect of temperature on post-mortem isometric tension; 3) suspension of strips in solution makes it possible to add agents that will prevent bacterial growth during the course of the experiments; 4) suspension of strips in solution makes it possible to investigate the effects of changes in pH, ionic strength, or chemical composition on post-mortem isometric tension. Consequently, all isometric tension measurements in this study were done on strips immersed in saline solution.

With this technique, it has been shown that isometric tension development and subsequent isometric tension decline occurs in post-mortem bovine, rabbit, and porcine muscle at all four temperatures studied. Usually, a

very rapid rate of isometric tension development is subsequently accompanied by a rapid rate of isometric tension decline. Failure of previous studies to observe isometric tension decline at temperatures above 10° was probably due to dehydration of the muscle strips in the earlier studies. Post-mortem isometric tension patterns of rabbit muscle resemble those of porcine muscle; maximal tension development occurs at 37°, and there is very little difference in amount of isometric tension developed at 2°, 16°, and 25°. However, rabbit muscle develops 2-3 times as much tension per unit cross-sectional area at 37° as does porcine muscle. Bovine muscle develops maximal isometric tension at 2°. Isometric tension development of bovine muscle is minimal at 16° and increases very slightly in the 25-37° range.

Changes in post-mortem isometric tension closely parallel post-mortem changes in length of isotonically suspended strips. However, changes of 50% in isometric tension are accompanied by changes of only 4-5% in length of isotonically suspended fibers. Moreover, lengthening of isotonically suspended fibers is not as complete nor as rapid as the corresponding isometric tension decline. Changes in extensibility of post-mortem muscle strips do not closely parallel changes in post-mortem isometric tension. Loss of extensibility does not occur until maximum isometric tension development has been reached and once post-mortem muscle has become inextensible it does not regain its extensibility. Thus there is no extensibility change corresponding to isometric tension decline. Consequently, isometric tension is the most sensitive and informative method for measuring physical changes in post-mortem muscle.

Changes in extracellular pH *per se* are not the cause of post-mortem isometric tension development and decline. Decreasing extracellular pH from 7.0 to 5.0 at 37°, increases the rate of isometric tension development and decline, but decreasing extracellular pH from 7.0 to 5.0 at 2° decreases the rate of isometric tension development and decline. Although previous studies have suggested that the sarcolemmal membrane prevents intracellular pH changes in response to changes in extracellular pH, it seems probable that the large variation in extracellular pH used in this study also caused some changes in intracellular pH and therefore that intracellular pH *per se* is not a cause for post-mortem isometric tension development and decline. This conclusion was substantiated by the finding that storage of myofibrils prepared from at-death muscle for 7 days at pH values ranging from 5.5 to 8.0 did not cause any change in the Mg^{++}-modified, the Ca^{++}-modified, nor the Mg^{++}-EGTA-modified ATPase activities.

Addition of trypsin to the bathing medium also did not affect the post-mortem isometric tension pattern of rabbit *psoas* muscle strips. Since trypsin would be expected to have a large effect on the post-mortem isometric tension pattern, it is probable that the sarcolemmal membrane is impermeable to trypsin, even after 5-6 hours post-mortem in the presence of 1mM deoxycholate. This result suggests that extracellular cathepsins probably do not have an important role in post-mortem tenderization.

Addition of Ca^{++} to the bathing medium did not affect the rate of isometric tension development but did markedly increase the rate of post-mortem isometric tension decline. Surprisingly, addition of 1mM EDTA to the bathing medium did not prevent post-mortem isometric tension develop-

ment, but actually appeared to potentiate it. Since EDTA chelates both Ca^{++} and Mg^{++}, and since both Ca^{++} and Mg^{++} are obligatory requirements for muscle contraction, it is almost certain that EDTA did not penetrate the sarcolemmal membrane. Furthermore, the sarcolemmal membrane must also be impermeable to Ca^{++} and Mg^{++} during the first 5-6 hours post-mortem, because Ca^{++} and Mg^{++} would otherwise diffuse out of the muscle cell and be chelated by EDTA. The Ca^{++} impermeability of the sarcolemma also probably accounts for the lack of effect of added Ca^{++} on rate of isometric tension development. Isometric tension decline in the presence of added EDTA is imperceptible, in contrast to the effect of added Ca^{++} on the isometric tension decline. This suggests that after 5-6 hours post-mortem, the sarcolemma becomes permeable to Ca^{++} and EDTA and permits them to exert some effect directly on the myofibrils.

Microscopic examination of samples from Ca^{++} and EDTA-treated muscle strips showed that the presence of Ca^{++} in the bathing medium caused a dramatic Z-line removal. The presence of EDTA, on the other hand, causes a remarkable preservation of Z-line structure in post-mortem myofibrils, even at 37^{o}. This effect was due to Ca^{++}-chelation by EDTA since EGTA, which chelates Ca^{++} but not Mg^{++}, also caused preservation of post-mortem isometric tension development and Z-line structure. When 1mM deoxycholate was included in the solution, between 3 and 9 hours were required for Z-line removal by Ca^{++}-treatment at 37^{o}. This time was extended to 24 hours when Ca^{++}-treatment was done at 2^{o}. After 24 hours at 37^{o}, the M-line and the thin filaments of myofibrils from Ca^{++}-treated fibers were intact.

This suggests that the effect of Ca^{++} is specifically on the Z-line.

Muscle strips consisting predominantly of red fibers develop greater post-mortem isometric tension than muscle strips consisting predominantly of white fibers. However, addition of 1mM deoxycholate to the bathing medium removes this difference between red and white muscle strips. Since the effect of deoxycholate is presumably on the sarcolemmal membrane, these results suggest that the sarcolemmal membranes of red muscle fibers differ from those of white muscle fibers.

CONCLUSIONS

1. Post-mortem isometric tension patterns of muscle strips suspended in saline solution are very similar to those of muscle strips suspended in humid air. However, the technique of suspending strips in saline solution has these advantages: a) dehydration frequently encountered at elevated temperatures in humid air is eliminated, b) bacteriocidal and bacteriostatic agents can easily be added to the solution to eliminate bacterial growth, c) temperature control is more precise, d) effects of varying pH, ionic strength, or chemical composition of the bathing solution may be tested with greater ease and reproducibility.
2. When measured on bovine, porcine, or rabbit muscle strips suspended in solution, post-mortem isometric tension development is always followed by a loss in ability to maintain isometric tension. This occurs at post-mortem temperatures of 2, 16, 25, and 37°. A rapid rate of isometric tension development is accompanied by a rapid rate of isometric tension decline.
3. Changes in isometric tension development and decline of post-mortem muscle strips closely parallel post-mortem changes in length of isotonically suspended strips, but changes of 50% in isometric tension are paralleled by changes of only 4-5% in length of isotonically suspended fibers. Loss of extensibility in post-mortem muscle does not occur until maximum isometric tension development has been reached, and there is no extensibility change that corresponds to isometric tension decline. Consequently, isometric tension is the most sensitive and informative method for measuring changes in post-mortem muscle.

4. Post-mortem isometric tension patterns of rabbit muscle resemble those of porcine muscle: tension development at 37° is double tension development at 25°, but there is very little difference in amount of tension developed at 2, 16, or 25°. Bovine semitendinosus muscle develops twice as much tension at 2° as it does at 37°, and there is very little difference in amount of tension developed at 16, 25, and 37°. Post-mortem isometric tension development of bovine muscle at 37° is about one half the isometric tension developed by porcine muscle at 37° and about one-fourth the isometric tension developed by rabbit muscle at 37°.

5. Changes in extracellular pH per se do not cause isometric tension development and decline in post-mortem muscle. However, rate of tension development and decline increases as pH is lowered from 7.0 to 5.0 at 37°. At 2°, rate of tension development and decline decrease as pH is decreased from 7.0 to 5.0. Storage of at-death myofibrils for 7 days at 2° and at pH values ranging from 5.5 to 8.0 has no effect on the Ca^{++}-modified, the Mg^{++}-modified, or the Mg^{++}-EGTA modified ATPase activities of the myofibrils.

6. Addition of Ca^{++} or EDTA to the bathing medium does not affect the rate of post-mortem isometric tension development, even in the presence of 1mM deoxycholate, but after 4-6 hours post-mortem, both Ca^{++} and EDTA have marked effects on isometric tension decline. The presence of Ca^{++} causes a very rapid rate of isometric tension decline whereas isometric tension decline is almost imperceptible in the presence of EDTA (absence of Ca^{++}).

7. Addition of trypsin to the bathing medium does not affect either post-mortem isometric tension development or decline, even in the presence of 1mM deoxycholate. Therefore, extracellular proteases probably do not play an important role in meat tenderness during the first 24 hours post-mortem.
8. The sarcolemmal membrane is not permeable to Ca^{++} or EDTA during the first 4-6 hours post-mortem and is not permeable to trypsin for at least the first 8 hours post-mortem.
9. Addition of Ca^{++} to the bathing solution causes complete Z-line removal, but the addition of EDTA causes a remarkable preservation of Z-line structure in post-mortem myofibrils even at 37^{o}. Myofibrils prepared from Ca^{++}-treated strips are very fragmented and only 2-3 sarcomeres in length, but myofibrils prepared from EDTA-treated strips are 8-10 sarcomeres in length. Between 3-9 hours were required for Z-line removal at 37^{o}. This time was extended to 24 hours when Ca^{++}-treatment was done at 2^{o}. The Ca^{++} effect is specifically on the Z-line, since both thin filaments and the M-line were intact after 24 hours of Ca^{++}-treatment at 37^{o}.
10. Porcine muscle strips consisting predominantly of red fibers develop more post-mortem isometric tension than strips consisting predominantly of white fibers. This difference is probably due to a difference in the sarcolemmal membranes of red and white fibers since addition of 1mM deoxycholate to the bathing medium removes the difference in isometric tension development between red and white muscle strips.

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