THE EFFECT OF DANTROLENE SODIUM ON POST MORTEM CHANGES IN RAT GASTROCNEMIUS MUSCLE

By

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CHAPTER I

INTRODUCTION

Tenderness is considered to be one of the most important quality characteristics of meat (Lawrie, 1970). With the advent of a new processing method for beef, hot processing, a problem with palatability has arisen. Relatively less tender meat results from muscle which is boned prior to the onset of rigor and subsequently chilled (Falk and Henrickson, 1974). Hot processing involves the removal of the musculature prior to the onset of rigor mortis, and prior to the cooling of the carcass (Pierce, 1977). When the attachment of the musculature to the skeletal framework of the animal is severed before rigor development, the muscles are free to shorten to a greater extent than muscles held rigidly in place on the carcass (Stouffer, 1978). The problem is enhanced when cold shortening is allowed to take place. Cold shortening is a process characterized by a marked fiber shortening which takes place in the unrestricted muscle when rigor is preceeded by a severe chilling of the tissue. The resulting meat is significantly more tough than that handled in a conventional manner (Marsh and Leet, 1966).

Hot processing has been shown to result in large energy

savings (Noble, 1977). The removal of the bones and some of the excess fat prevents the need for cooling of these waste products (Kastner et al. 1973). Henrickson (1975) proposed a savings of up to 50% in refrigeration capacity as well as additional energy savings in transportation of the more compact lean muscle mass. In addition, hot processing allows more of the muscle tissue to be utilized as higher priced steaks and roasts than is presently possible with conventional methods (Noble, 1977).

To allow the bovine hot processed muscle to be used as higher quality cuts, the tenderness problem had to be overcome. At present, electrical stimulation of the carcass is the most effective and practical method of expending tissue energy. McCollum (1977) observed a quick drop in the pH of stimulated muscle which corresponded to an accumulation of lactic acid in the tissue. He suggested that the loss of adenosine triphosphate (ATP) due to the electrically induced contraction deprived the muscle of the energy source needed for further contraction. This allowed the tension on the muscle to be fixed shortly after stimulation and resulted in an induced rigor state.

Pierce (1977) found that the sides of beef stimulated were significantly more tender than those processed without stimulation. Will (1978) indicated that electrical stimulation may cause some damage to the microstructure due to the severe contraction involved. This may explain why the resulting meat appears to retain less moisture during cooking.

Another possible method of decreasing the toughening effect of hot processed muscle would be to prevent the formation of rigor. Streital et al. (1977) found that the addition of calcium chelating agents such as sodium pyrophosphate and sodium hexametaphosphate resulted in a significantly more tender product. Although the inhibition of rigor by infusion of chelating agents is possible, the treatment is impractical for large peices of meat or when rapid processing is necessary.

The purpose of this study was to investigate the effect of a skeletal muscle relaxant, 1-(5-(p-nitrophenyl) furfurylidene) amino hydantoin sodium hydrate (dantrolene sodium) on some of the chemical and physical properties of post mortem muscle.

CHAPTER II

LITERATURE REVIEW

The effect of dantrolene sodium is in lessening the tension development of living muscle during contraction. It was felt that in order to understand its possible effects in post mortem muscle a brief description of some aspects of muscle contraction and rigor development should be included.

Sarcoplasmic Reticulum

Excitation

Following excitation of the neuromuscular junction and transmission of this signal through the T-tubular system the sarcoplasmic reticulum (SR) is activated. Franzini-Armstrong (1970) described an area between the T-system and the SR which may provide the area of physical connection between these systems. This area is bridged by structures referred to as sarcoplasmic reticular feet. Constantin (1975) suggested that these feet might be able to accomodate the transfer of small ions such as potassium ions causing some electrical depolarization of the SR. Using voltage-clamp measurements of frog muscle, Schneider and Chandler

(1973) obtained data supporting a model in which a fixed amount of charge is free to move across the membrane. They further stated that the electrical potential is in a range not unlike many of the electrical and mechanical properties of muscle with current sufficiently small to prevent interference with normal action potentials. Schneider and Chandler speculated that charged movement could be the means by which the SR "senses" the T-system action potential or could be the mechanism directly regulating the release of calcium ions (Ca++) from the SR.

Calcium Release

Following the events which lead to the transmission of a voltage dependent charge movement between the T-system and the SR, the SR loses part of its load of Ca++ and contractile activation is initiated. The SR is, according to Hasselbach (1977, p. 43), "a highly specialized structure into which only two major intrinsic proteins, a Ca++ transport protein and a Ca++ binding protein are embedded". In mammalian muscle, the SR forms large cisternae which combine with the T-system to form structures known as triads. These triads lay on the junction of the A and I bands and thus are in position for maximum utilization of their Ca++ content during contraction.

Immediately following excitation-contraction coupling, the SR lateral cisternae release large amounts of Ca++ resulting in an increase in the extra-reticular Ca++ concen-

tration from a resting concentration of 10^{-7} mM to a concentration of 10^{-6} mM or greater needed for contraction (Constantin, 1975). There is considerable disagreement concerning the method of Ca++ release from the SR. In general, most research has favored either Ca++ induced-Ca++ release (CICR) or depolarization induced-Ca++ release (DICR).

At room temperature, CICR requires a Ca++ load above physiological levels to induce further release (Taylor and Godt, 1976). Any of the following conditions can induce CICR: (1) addition of caffeine at a concentration of 2 mM, (2) increase in Ca++ concentration to 10^{-4} mM, (3) decrease in temperature, or (4) superloading of the SR with Ca++ (Ebashi, 1976). The graded release of Ca++ seen in physiological situations is one of the major problems with CICR theory. It has been reported by Endo and Kitizawa (1976) that in the absence of ATP, CICR is depressed. It is possible that other factors such as pH or ionic strength may function as CICR modifiers.

Depolarization induced-Ca++ release appears to be a more probable mechanism of SR/Ca++ release, but still possesses one major difficulty. Equipment required for direct measurement of ionic change within the SR has not yet been developed. Use of labeled ^{45}Ca++ by Stephenson (1975) demonstrated that ionic exchange does result in concentration due to the release of Ca++ from the SR. Even if Ca++ release occurs because of ionic exchange, the possibility exists that some effect other than depolarization

such as shrinkage or swelling of the SR may be the cause of the observed Ca++ release.

Kitizawa and Endo (1976) suggested that once the membrane is effected, whether it be by CICR or DICR, Ca++ passes through the SR membrane by simple diffusion due to changes in membrane permeability.

Calcium Induced Contractile Activation

Following the release of Ca++ into the myoplasm, the actin and myosin filaments begin the process of forming actomyosin cross-bridges resulting in the physical shortening of the fiber called contraction. As early as 1963, Davies at the University of Pennsylvania gave a detailed account of the "sliding filament theory" of muscle contraction (Davies, 1963). When Ca++ released from the SR reaches a myoplasmic concentration of 10^{-6} mM protein interaction is initiated. Working with skeletal muscle fibrils, Fuchs and Briggs (1968) demonstrated that myofibrils possess one location of major Ca++ binding ability at physiological Ca++ levels. The site is troponin and more specifically, one troponin segment called TN-C. When TN-C complexes with Ca++ a change in the conformation of the troponin takes place which alters the position of the tropomyosin molecule. This results in the removal of the inhibitory effect of tropomyosin on the actin-myosin interaction (Collins, 1976). Figure 1 illustrates the relative changes which occur as a result of Ca++ binding to TN-C (Cohen, 1975).

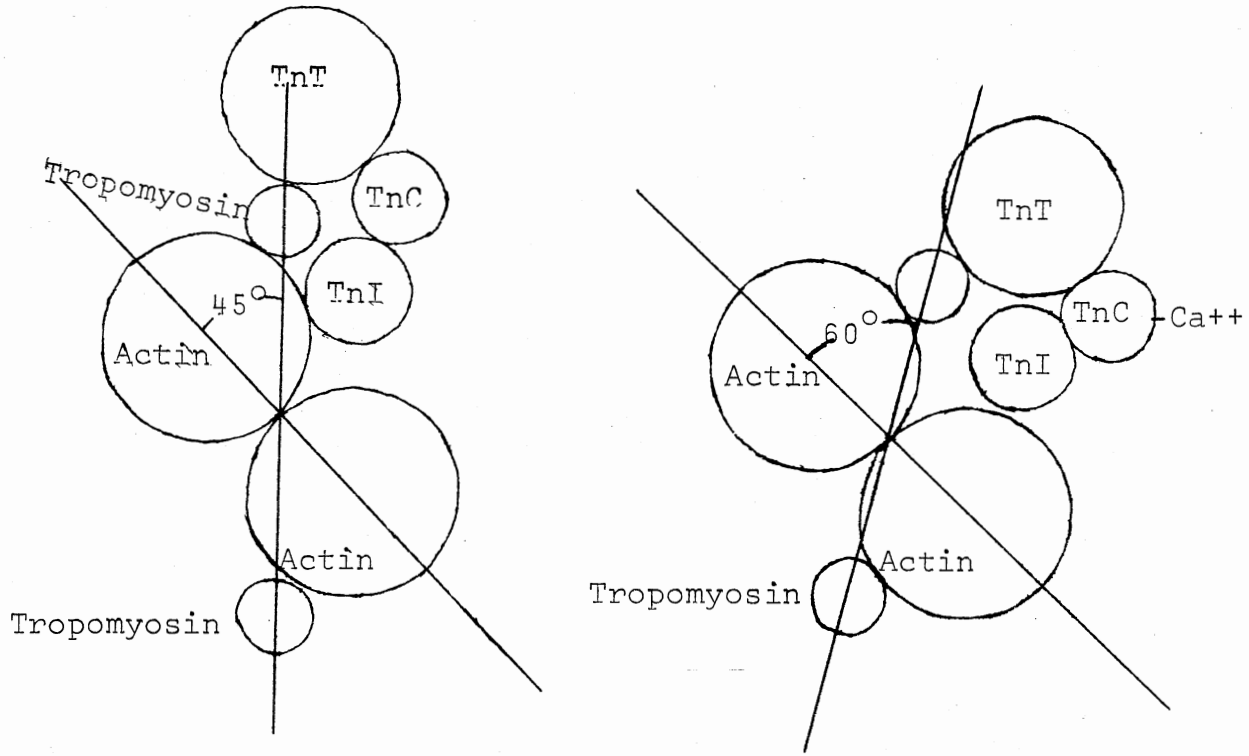


Figure 1. The Shift in Tropomyosin Due to the Effect of Troponim and Calcium

For contraction to occur, adenosine triphosphate - magnesium ion complex (ATP-Mg++) is required. This ATP is broken down to ADP releasing energy which enables the "power-stroke" or rowing motion of the myosin head (Guyton, 1976). However, in the presence of ATP, dissolution of the actomyosin occurs (Koretz et al., 1972). Using purified muscle proteins, Crooks and Cooke (1977) demonstrated that the ATP effect occurred with or without troponin or tropomyosin. They suggest that the energy derived from splitting ATP resulted in a breakage of the actin and myosin linkage.

Rigor Mortis

Chemical Changes

Upon exsanguination, many changes occur in muscle, the most noteable of which is the breakdown of the body's energy stores to lactic acid. Muscle continues using ATP for power-stroke movement, the splitting of actomyosin, and to a smaller extent for Ca++ uptake by the SR. With the removal of an oxygen source, respiration, the transformation of glycogen and glucose to carbon dioxide via aerobic glycolysis ceases, and anaerobic glycolysis or the breakdown of glycogen to lactic acid begins. Arnquist and Lundholm (1976) using mesenteric arteries demonstrated that a reduction in the oxygen environment from 90 to 20% caused little change in metabolism, but a reduction from 20 to 0% oxygen resulted in a four-fold increase in lactate production. They

noted marked decreases of glycogen, creatine phosphate, and ATP at 0% oxygen levels. Sacks and Morton (1966), using Cat gastrocnemius muscle found that occluded blood supply resulted in a slight increase in pyruvate concentration with concurrent large increases in lactate concentration.

The reactions of aerobic glycolysis result in the formation of 36 M of ATP per mole of glucose as opposed to 2 M of ATP formed from anaerobic glycolysis. The lowered production of energy from equivalent amounts of fuel leads to quicker utilization of available sources of energy. Sahlin et al. (1975) reported an increase in the ratio $\frac{(\text{creatine})\ (\text{ATP})}{(\text{CP})\ (\text{ADP})}$ from 4.62 at rest to 9.59 after 15 minutes in occluded quadriceps muscle. The breakdown of glycogen to lactic acid produces 4.55 kJ of heat per kilogram body weight (Bendall, 1973). An actual heat production of 4.5 kJ per kilogram in bovine semitendinosis muscle, was felt by Morley (1974) to suggest that anaerobic glycolysis was the only energy production mechanism in muscle after death.

Along with, or because of, an increase in lactate content, a decrease in the pH of muscle is seen post mortem. Hermanssen and Osnes (1972) showed a decrease in human tissue pH from 6.92 to 6.41 after maximal exercise and blood occlusion. Sahlin et al. (1975) found a drop in human pH from 7.09 at rest to 6.56 following circulatory blockage and found that the drop in pH was linearly related to the combined accumulation of pyruvate and lactate.

Physical Effects

Contraction associated with rigor mortis resembles normal muscle contraction in many respects. In rigor, actomyosin cross-bridges form, the sarcomeres shorten, and Ca++ causes a change in the troponin-tropomyosin configuration allowing contraction to occur. However, other attributes of rigor are very different from muscle contraction. In rigor, the actomyosin generated does not power-stroke or dissociate due to the splitting of ATP, and Ca++ may or may not be present for contraction.

Rigor inelasticity begins when muscle ATP stores and ATP precursors are exhausted (Nauss and Davis, 1966). It is postulated that the fairly permanent bond formed during rigor is an electrostatic interaction between the positive ATP binding site on the heavy meromyosin and the negative ADP bound to F-actin (Davies, 1963).

The presence of Ca++ does not appear to be necessary for the formation of rigor contraction (Weber and Murray, 1973); however, in bovine muscle, post-rigor tension has been shown to be affected by removal of Ca++ from the system pre-rigor (Streitel et al., 1977). They found that the use of sodium hexametaphosphate, a Ca++ sequestering agent, increased meat tenderness. Feinstein (1966) exposed frog muscle to two Ca++ chelators, ethylenediamine tetraacetate and ethyleneglycol bis (B-aminoethlether)-N,N'-tetraacetic acid, and found that both the time course of rigor and the

ultimate magnitude of the contracture were inhibited.

Bremel and Weber (1971), Bremel and Weber (1972) and Weber and Murray (1973) found that the actin filament was turned on in the absence of Ca++ when a certain number of actin monomers combined with myosin. These "forbidden interactions" regulate rigor contraction. In uncontracted muscle, the angle of tropomyosin from the axis of the actin dimer is 45°. According to Squire (1975) this is an average value and a variation of +/- 5° in living tissue can be anticipated. An oscillation to a 50°angle could result in an actin position which is inactive but not inhibited. A myosin fragment would then be free to associate with the actin site and may even push the tropomyosin molecule in close proximity to the actin activating location.

Dantrolene Sodium

In 1967, a new group of skeletal muscle relaxants were synthesized at the Norwich Pharmacol Company (Snyder et al., 1967). One member of this class, 1-(5-(p-nitrophenyl) furfurylidene) amino) hydantoin sodium hydrate or Dantrolene Sodium (DS) proved to be safe and effective for the treatment of spasticity due to disease or injury.

Uses of Dantrolene Sodium

Dantrolene sodium is effective in the treatment of spasticity due to hemiplegia (Steinberg and Ferguson, 1975), multiple sclerosis (Gelenberg and Poskanzer, 1973; Liver-

sedge, 1977), cerebral palsy (Soboloff, 1974; Denhoff et al., 1975), and in the treatment of spasticity due to trauma and injury (Knutsson and Martensson, 1976; Schmidt et al., 1976). According to Dykes (1975), Dantrolene is useful for spasticity therapy because it reduced pain, increased patient usage of existing motor ability, and increased the usage of rehabilitative devices.

In animals, the most extensive use of DS has been for the treatment of Malignant Hyperpyrexia in swine. Malignant Hyperpyrexia (MH) is a genetic disease which results in stiffening and death to MH prone pigs that are exposed to a stress or Halothane anesthesia (Harrison, 1975). Meat produced from these pigs has a tendency to be soft, watery and pale colored. Addition of DS has been shown to give significant protection to stress suseptible pigs (Anderson and Jones, 1976; Harrison, 1975; Harrison, 1977). Gronert et al. (1976) treated stress suseptible pigs with DS at a rate of five mg per kilogram body weight before exposure to Halothane gas, 7.5 mg per kilogram after exposure to Halothane. Treatment prior to Halothane resulted in complete inhibition of MH symptoms, treatment after exposure resulted in a rapid reversal of the MH symptoms, but no treatment resulted in full development of MH symptoms and eventual death.

Metabolism and Effective Dosages

Dantrolene sodium can be administered orally in capsule form, or injected intraperitoneally or intravenously. An

effective intraperitoneal dose needed to noticeably effect muscle contraction in mice was 10 to 20 mg per kilogram while in rats 25 mg per kilogram was required (Ellis et al., 1973). The peak effect of DS was seen one hour after injection, and the duration was dose dependent from a dose of 200 mg per kilogram lasting longer than 8 hours to 400 mg per kilogram lasting over 18 hours.

Dantrolene administered orally is absorbed in the small intestine. It has been estimated that 80% of the DS absorbed is excreted as 5-hydroxydantrolene, 17% is excreted as an acetamino derivative, and 1-4% is excreted unchanged (Pinder et al., 1977). The 5-hydroxy form also has skeletal relaxing abilities although much lower than DS (Snyder et al., 1967).

Safety and Side Effects

In considering any drug used to inhibit muscular contraction, an investigation into its safety for long-term usage is critical. The most obvious question would deal with the effect of the drug on cardiac and smooth muscle types (specifically the heart and lungs). Drugs such as curare, mephenesin, and benazole used to control spasticity act on neural or neuromuscular targets. The inhibition of the respiratory and cardiac function which this entails places extreme limitations on their usage. Meyler et al. (1976) found that DS gave a dose-dependent decrease of up to 74% in rat diaphram muscle activity. A dose-dependent decrease in rat heart muscle of up to 74% was also seen.

Bowman and Kahn (1977) however, found no depression of cardiac or smooth muscle at a concentration able to cause 50% depression of the soleus muscle twitch, but higher concentrations did produce some inhibition. These findings disagree with those of Ellis et al. (1975), Ellis (1967a), and Ellis et al. (1976b) in which no inhibition was seen in vivo in dogs or sheep cardiac or respiratory function. A warnings against use by patients with severe pulmonary difficulties was advised by Bowman and Kahn (1977), but when used with animals the degree of cardiac problems necessary to require care in the use of the drug is unlikely to be encountered.

Another area of danger in the use of any drug over the long run is the effect the drug has on the organ which eliminates it from the body. Joynt (1976), Haslam et al. (1974), Pinder et al. (1977), and Assatourians (1976) did report some hepatic damage associated with the use of high levels for long periods of time.

Dantrolene usage reportedly causes a number of side effects such as dizziness, weakness, inebriation, diarrhea, nausea, constipation, and headaches, with weakness and drowsiness the most common (Chayette and Basmajian, 1973; Denhoff et al., 1975; Lietman et al. 1974). However, Chayette and Birdsong (1971) found that side effects usually lasted only 24 to 48 hours and that patients receiving placebo treatment also reported a significant number of side effects.

Site of Muscular Action

Ellis et al. (1973) and Odette and Atwood (1974) found the effect of DS to be beyond the neuromuscular junction. Using data based on work with rat diaphram muscle, Ellis and Bryant (1972) concluded that DS produced a direct inhibitory effect on skeletal muscle. They implicated either inhibition of the actin-myosin coupling or more probably a decrease in the amount of Ca++ released from the SR. That DS has its effect on a decrease Ca++ efflux from the SR was confirmed by Monster et al. (1974), Ellis and Carpenter (1974) and Brockelhurst (1975). Putney and Bianchi (1974) and Takauji et al. (1975) were early proponents of a T-system-SR interaction because of a possible effect on trigger Ca++. Later research has supported the original idea that the effect of DS was on the SR/T-system junction, but has determined that inhibition of a charged particle movement was more probable than inhibition of a trigger Ca++ (Morgan and Bryant, 1977; Van Winkle, 1976). Extensive study of Ca++ movement in giant barnacle muscle fibers by Desmedt and Hainaut (1977) provided strong evidence that DS inhibited electrochemical coupling and reduces the proportion of Ca++ releasing sites which can be activated per depolarization.

Propylene Glycol

One undesirable characteristic of DS is its relative insolubility in water. Water will solubilize DS at a rate

of less than 1 mg per liter (Ellis, 1977). Propylene glycol has been used for many years as a carrier agent for drugs which possess low solubility rates in water. Dantrolene will dissolve in propylene glycol up to 40 mg per milliliter.

Propylene glycol because of its low toxicity was considered to be an ideal carrier (Lehman and Newman, 1937). It was formerly thought to be essentially inert. Zaroslinski et al. (1971) proposed that the use of propylene glycol in intraperitoneal injections not be recommended as it functions as a weak, central nervous system depressant. Bonnardeaux (1971) suggested that propylene glycol acts as a depressant of the metabolism of isolated rat uterine muscle decreasing frequency and tension of muscle contraction.

Propylene glycol is metabolized in the liver, ultimately forming pyruvate and lactic acid, suggesting a possible function as an energy source (Hanzlik et al., 1939). Wittman et al. (1975) indicated that administration of propylene glycol resulted in a 7 mg% increase in blood glucose in fasting rats. This was attributed to PPG acting as a substrate for glucose formation.

Essentially therefore, propylene glycol appears to have two effects in muscle: (1) it decreases muscular activity and (2) it increases the amount of energy source available to the muscle.

Differentiation of Myofiber Types

Skeletal muscle fibers vary in their relative abilities to obtain energy by glycolysis or by oxidative phosphorylation. Classification schemes for fiber type range from two classes (red or white; type I or type II), to as many as eight individual groups. A classification method consisting of three classes of fibers (red, white, and intermediate) is most commonly used. Ashmore and Addis (1972) described the red fibers as those slow acting fibers which contain large quantities of myoglobin and form energy by oxidative phosphorylation. The fibers possess an extensive blood supply and quickly remove waste products allowing for long-term activity. Close (1972) described white fibers as quick acting, quick tiring fibers which depend upon anaerobic glycolysis for energy production. Beatty et al. (1963) showed a higher initial glycogen level in white fibers accompanied by a much lower glycogen level two hours postmortem. Although the two hour ATP-ADP levels were essentially equal in both types of fibers, the lactate produced was significantly higher ($P < 0.005$) in the white fiber.

The relative slowness of red fibers in both metabolic function and tension development makes them valuable for use in studies of rigor formation. Several methods have been used to determine fiber type of skeletal muscle. The most commonly used methods are staining techniques for oxidative (Succinic Dehydrogenase; Nicotinamide Adenine

Dinucleotide - Reduced form, (NADH) or for glycolytic (Adenine triphosphatase) enzymes. Jobsis and Stainsby (1968) demonstrated the oxidation of NADH to NAD in dog muscle under maximal twitch. Cooper et al. (1963) used NADH-Tetrazolium stain (NADH-TR) on skeletal muscle and used the reduction of NADH-TR activity as criterion to demonstrate an increase in white fiber type with age.

The reaction of NADH-TR was described by Scarpelli et al. (1958). Basically, the added NADH donates electrons to NAD located in the cell which transfers the electrons to the tetrazolium salt being used (commonly, Nitroblue Tetrazolium Salt). The electrons transferred to the tetrazolium salt transform the salts to formazan. As a result, in the positions where NAD is present deposits of dark blue formazan are formed. The red, intermediate, and white fibers react variable according to the amount of NAD present. Red fibers contain the most NAD oxidative enzyme while the intermediate and white fibers contain lower levels respectively.

CHAPTER III

MATERIALS AND METHODS

Muscle Selection

Muscle groups from five rats were utilized in a preliminary study to develop technique and to determine which muscle to use in the main study. The muscle systems used were the gastrocnemius and flexor digitorium herein called the gastrocnemius group (GA), the gluteus medias and gluteus profundis called the gluteus group (GM), the rectus femoris muscle (RF), the biceps femoris (BF) and the triceps brachii (TB). The Sprague-Dawley female rats were anesthetized using a bell jar containing ether-soaked cotton. Decapitation was performed and the animal was skinned, eviscerated, and washed to remove excess blood. The target muscles were excised, wrapped in aluminum foil and immediately frozen in liquid nitrogen. The total time which expired from decapitation to freezing was approximately 30 minutes.

Later, duplicate sections, 16μ thick, were made from each muscle using a Slee Cryostat microtome. The sections were dried on microscope slides for 30 minutes and incubated

at 37°C for an additional 30 minutes in a media prescribed by Engle and Brook (1966).

0.2 M Tris buffer	20 ml
Nitroblue Tetrazolium	20 mg
Nicotinamide Adenine Dinucleotide (Reduced form)	16 mg

The slides were rinsed in sequence in solutions containing the following ratios of alcohol to water:

30/70, 60/40, 90/10, 60/40, 30/70

Each slide was then rinsed in distilled water and a coverslip mounted using Permount diluted with distilled water. The slide was then examined with a light microscope, 100X power, to determine the relative proportion of alpha and beta type fibers present. Evaluation of the muscles as to size, fiber orientation, and ease of removal was also made.

Experimental Design

For the main experiment, 48 Sprague-Dawley female rats weighing between 100 and 150 g were arranged into three, four by four latin squares. The vertical placement of the rats was determined according to pre-experiment weight with the heavier rats on the top shelf, and progressively lighter rats farther down on the rack. The columns were designed to measure the variation due to the relative position on the cage shelf.

Ground Purina Dog Chow eaten ad libidum made up the

core diet fed to the rats. In addition, once each day, each rat received 0.5 g ground veal. (The dog chow was removed during this period and was returned only after all the rats had completely consumed the veal.) Each cage was placed on a flat, papered shelf to prevent food loss through the wire mesh bottom of the cage.

Four treatments were applied to each row of the latin square (Figure 2). Treatment A consisted of feeding DS mixed in the 0.5 g veal at a dose of 15 mg per kilogram rat body weight for 30 days prior to slaughter. There was marked discrimination against eating the drug-treated veal. To control any loss of drug, it was manually mixed into the veal, so as to completely adhere to the veal. Rats on treatment B received plain veal for 30 days. On the thirty-first day, they were injected intraperitoneally with 1, 2-propanediol (propylene glycol) at a rate of 10 ml per kilogram body weight. The injection was given 1.5 hr prior to slaughter. (This was designed to be a control to measure the effect of stress and 1, 2-propanediol which was used as a carrier agent for the DS injection.) For treatment C, rats received veal for 30 days. These rats were the feed control group and received no injection prior to slaughter. Rats on treatment D were fed untreated veal and on the thirty-first day were injected with a DS/1, 2-propanediol solution intraperitoneally. One and one-half mg of DS was mixed per ml propanediol which was injected at a rate of 10 ml per kilogram body weight. This resulted in an

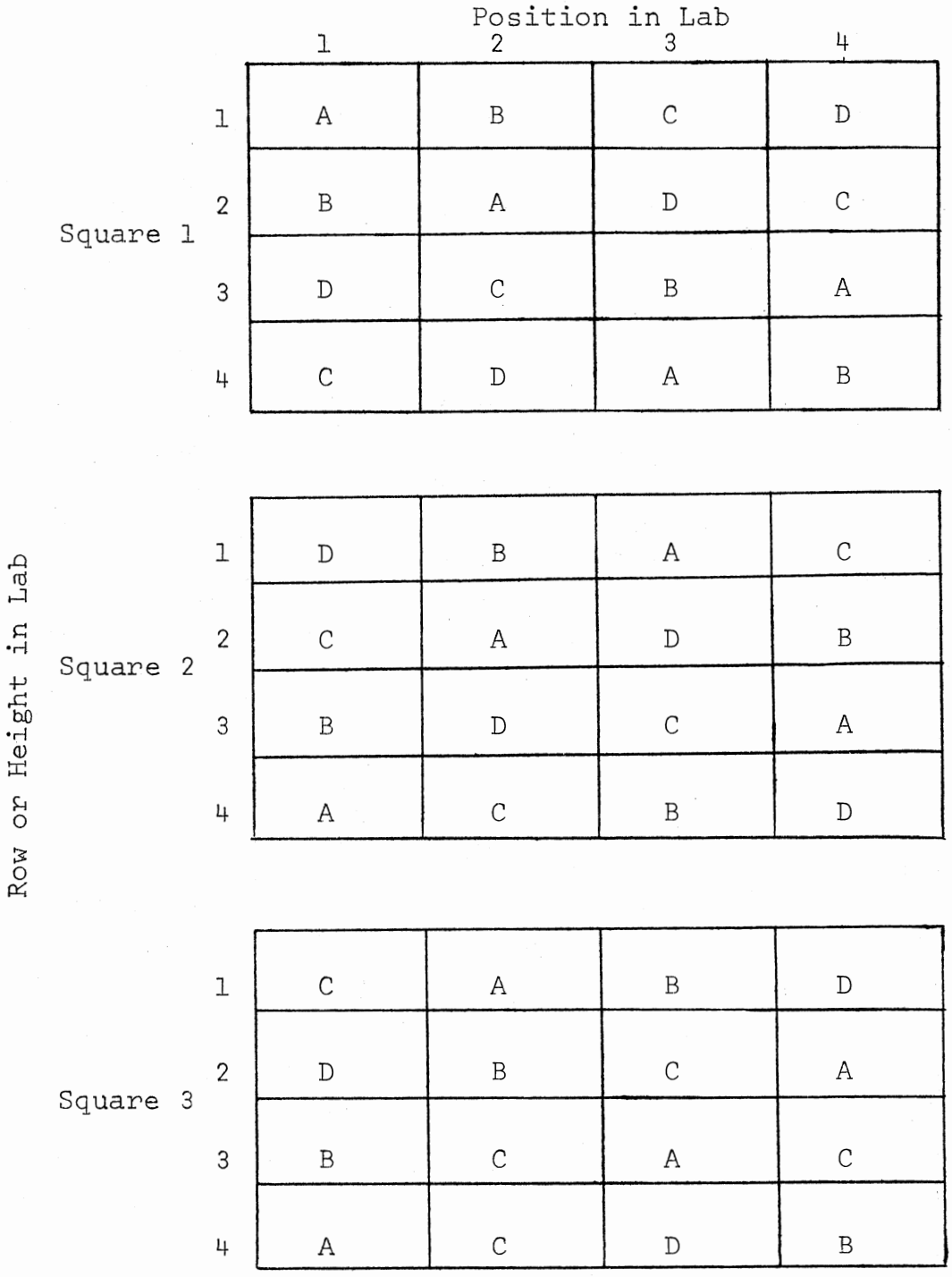


Figure 2. Placement of Rats in Latin Squares by Treatment

effective dosage of 15 mg per kilogram DS which was identical to the daily oral dosage. This injection was administered 1.5 hr prior to slaughter to ensure adequate distribution throughout the body. Table I illustrates the treatments presented to each group.

Each row of rats was started on test on consecutive days so that only four animals required tension measurements on a given day.

Tension and Time Measurements

The two rats which received injections were examined first each day. Two of the rats were anesthetized, using ether just to the point of unconsciousness and injected with approximately two ml of the appropriate drug combination. (Care was taken not to over anesthetize so as to avoid death.) The injection had a definite effect on the subsequent behavior of the rat causing it to remain inactive on the floor of the cage.

At the end of the 1.5 hr waiting period, one rat was reanesthetized and removed to a table for excision of the GA muscle. A nose cup consisting of a 50 ml beaker and ether-soaked cotton was used to maintain sleep. The first step involved splitting the skin over the left femur area. The skin was pulled away from the musculature exposing the entire leg. A small incision was then made through the BF muscle just proximal to the GA. A pair of surgical scissors helped to extend the cut longitudinally along the full

TABLE I

TREATMENTS DURING THE 30 DAY FEEDING PERIOD AND ON THE KILL DATE

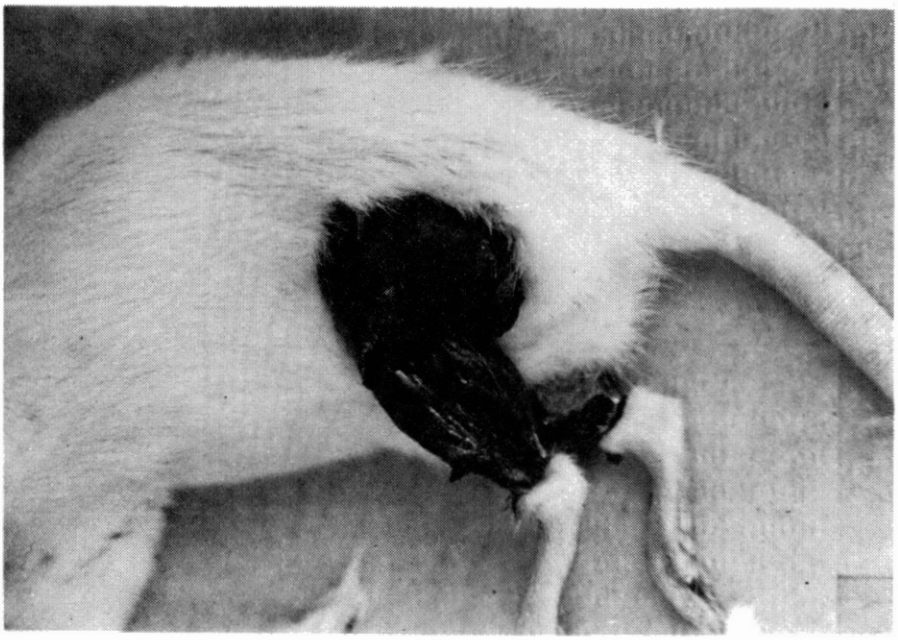
Treatment	30 Day Feeding Period	Test Day
A	0.5 g veal daily and 15 mg/kg rat weight dantrolene	0.5 g Veal and 15 mg/kg dantrolene
B	0.5 g veal daily	10 ml/kg intraperitoneal injection of 1, 2-propanediol
C	0.5 g veal daily	0.5 g veal
D	0.5 g veal daily	10 ml/kg intraperitoneal injection of 1, 2-propanediol - dantrolene solution 1.5 mg dantrolene/1 ml 1, 2-propanediol

length of the GA. The BF was gripped with forceps and pulled away from the GA taking care not to rupture the femoral artery (Figure 3a). A blunt probe was inserted between the Achilles tendon and the tarsals of the leg and pulled along the entire length of the tibia separating the body of the GA from the bone. At this point, an incision was made over the right femur and the some process was carried out on the right leg.

As soon as both muscles were free of attachment everywhere but at the origin and insertion, the process of tying off the left GA was begun. A piece of surgical silk approximately six inches long was passed between the Achilles tendon and the tarsals using a halfmoon needle. The silk was looped and tightly tied around the tendon. Another piece of silk was looped around the body of the muscle as near to its insertion as possible. The string was then pulled tightly and knotted (Figure 3b). (It is important to note that this is the first restriction of blood flow to the muscle.) The tendon distal to the first loop and the muscle bundle proximal to the second loop were then cut, freeing the muscle from the leg. (The excision process described was identical for all muscles.)

The left muscle was attached at the proximal end (muscle bundle) to a 100 g dead weight (Figure 4a) and lowered into a 250 ml beaker containing a modified Kreb's solution. The modified Kreb's solution consisted of 118 mM sodium chloride, 4.7 mM potassium chloride, 1.18 mM hydrated

A



B

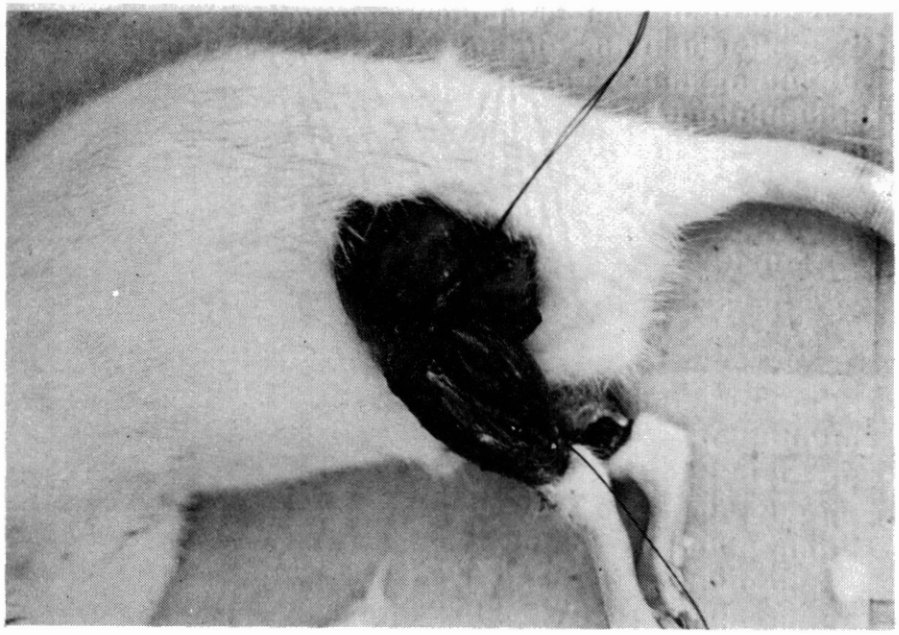
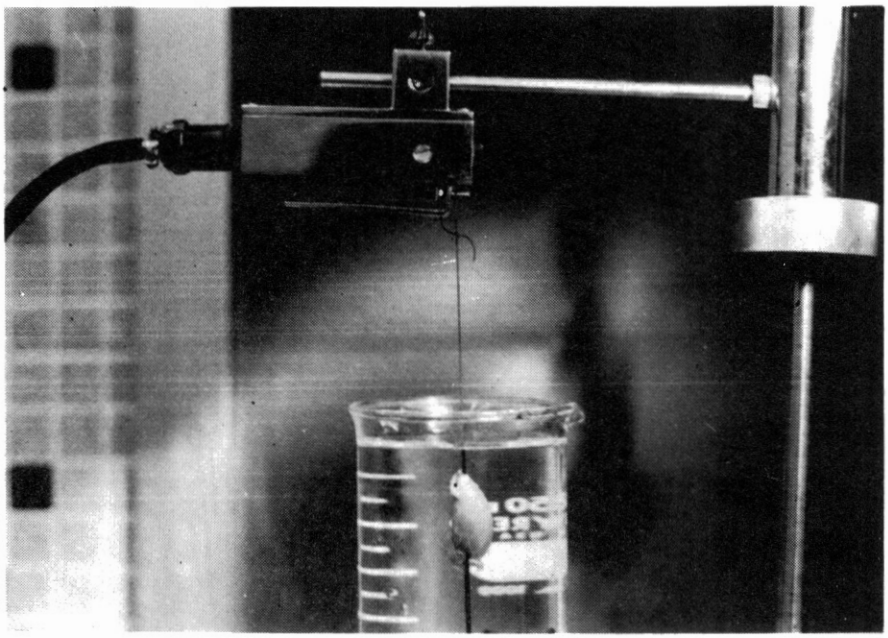


Figure 3. Removal of the Rat Gastrocnemius Muscle

A



B

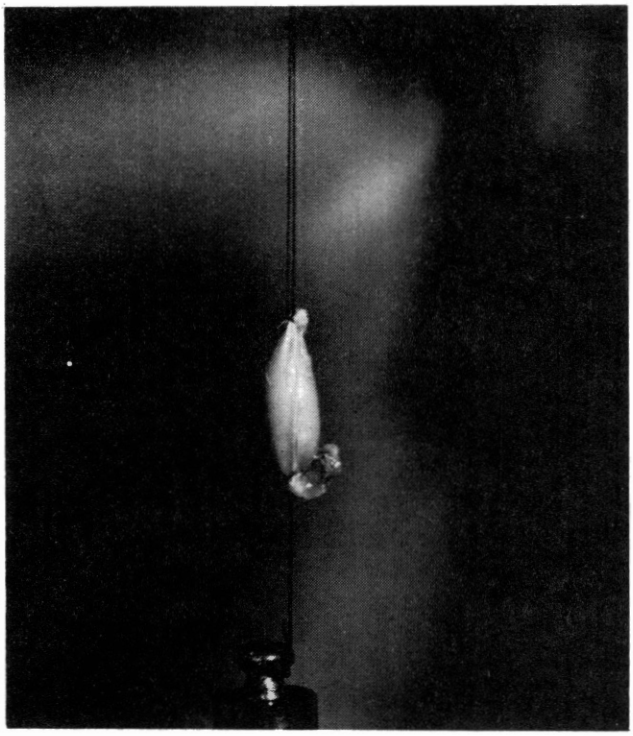


Figure 4. Attachment of the Gastrocnemius to the Type C Myograph and to the 100g Weight

magnesium sulfate, 1.2 mM monobasic potassium phosphate, 1.9 mM calcium chloride, and 25 mM sodium bicarbonate. (Regular Kreb's solution also contains glucose as a nutrient source. In this case, conditions resembling death were required resulting in the removal of the energy source.) The silk attached to the tendon was tied over the arm of a type C Myograph (Figure 4b). The myograph was attached to a physiograph via a transducer extension cable, a type 7173 transducer coupler, and a type 7000 channel amplifier. (Before attaching the muscles for each test, the physiograph was calibrated using a two and a five g weight.) Tension on each muscle of four g was drawn using a myograph tension adjustor. The adjustor used a concentric vernier control for fine adjustment of applied tension. The starting time was marked by a built in event marker which thereafter recorded each minute. The total time from the start of the operation to the first measurement was approximately 11 minutes and from the time blood flow to the muscle was cut off, to the time of tension measurement was one to two minutes. The right muscle was tied and excised in the same manner as the left and suspended in another beaker of modified Kreb's solution. No tension measurements were taken of this muscle. The time between suspension of the left muscle and the suspension of the right muscle was two to three minutes. The rat was then decapitated.

The second rat was treated in the same manner immediately following the death of the first. The time which

elapsed between the start of recording tensions on the first rat and start of recording on the second was typically between 12 to 16 minutes. (This was taken into account when recording values.) Four animals were treated in this manner each day with the injected rats being done as one pair and the fed rats as another. The assignment of kill order and physiograph channel selection for recording were done at random.

"Rigor mortis" was defined for this experiment as a 20 minute period after the second period of tension increase during which the muscle tension did not increase. (Pre-experiment observations using rat gastrocnemius muscles recorded on the physiograph indicated two characteristically sharp increases in muscle tension with a period of relative inactivity between them, and no subsequent increase in tension.) When the control or experimental muscles were determined to be rigor, the muscle not being recorded was wrapped in aluminum foil and frozen in liquid nitrogen for subsequent pH determination. The muscle being measured for tension was also wrapped in foil and frozen for later lactic acid tests.

The myographs were calibrated so that a one gram increase in tension caused a pen deflection of 0.5 cm. The recording paper being measured in 0.5 cm square boxes enabled easy calculation of tension development. Tension data was analyzed at 1, 2, 3, and 4 hours, as well as, the time the muscle was determined to be in rigor.

pH Measurement

The muscle was homogenized in 10 parts distilled, deionized water using a Kinematic Polytron homogenizer fitted with a three-quarter inch head. The pH of the homogenate was measured using a Corning Model 12 research pH meter fitter with a Metrohm combination electrode. The meter was calibrated before each group of muscle was measured with pHydrion standard buffers of pH 4.20 and 7.20.

Lactic Acid

Lactate (+) L content of each muscle was measured using the method reported in Standard Methods for Enzymatic Analysis (Bergmeyer, 1974). The muscle samples were homogenized with five ml of perchloric acid in centrifuge tubes using the Kinematic Polytron homogenizer. Additional perchloric acid was then added until the ratio of milliliters perchloric acid to grams tissue was 8 to 1. This was capped, swirled, and centrifuged in a Sorvell RC2-B centrifuge at room temperature for ten minutes at 3000 x gram.

Eight milliliters of this extract was combined with 0.02 ml methyl orange indicator in an ice-water bath. The methyl orange indicator consisted of 50 g methyl orange dissolved in water and made to 100 ml volume with additional water. While being magnetically stirred 0.1 ml at a time of carbonate solution was added until the color changed to a salmon-pink. The carbonate solution was made by dissolving 69 g

potassium carbonate in water with additional water added to 100 ml. After an additional 10 minutes in the bath to settle out the residue, 0.2 ml of the supernatant fluid was added to a tube containing 2.50 ml hydrazine/glycine buffer at pH 9.0. This buffer was made by dissolving 11.4 g glycine and 25 ml hydrazine in 200 ml of water and adding water to 300 ml. To the fluid and buffer was added 0.20 ml nicotinamide adenine dinucleotide solution. This solution was formed by dissolving 150 mg nicotinamide adenine dinucleotide in five ml of water. For the blank, 0.20 ml of perchloric acid was substituted for the supernatant fluid. The NADH formed from the reaction of lactate and NAD was measured using a Gilford 240 spectrophotometer at a wavelength of 340 nanometers with a blue filter. After the initial measurement, 0.02 ml of lactic acid dehydrogenase made from rabbit muscle was added to each tube. The lactic acid dehydrogenase was contained in a crystalline suspension in 3.2 molar ammonium sulfate with an activity of 800 units per milligram protein. The tubes were mixed using a vortex mixer, incubated in a 37^{o}C water bath for 30 minutes and remeasured. The initial reading was subtracted from the second reading and multiplied by 2.35 to obtain the lactic acid concentration in micromoles per milliliter in the tissue supernatant fluid. This was then multiplied by 8 (milliliters per gram) to obtain the total lactic acid concentration per gram of muscle.

Statistical Analysis

Analysis of Variance

All data were analyzed using the analysis of variance contained in the Statistical Analysis System (SAS) 76 programming system. The treatments were analyzed for their effect on tension at 1, 2, 3, and 4 hours and tension, pH, time to rigor, and lactic acid concentration were measured at rigor. Rat weight gain was also examined to investigate the effect of the feeding of the drug. The experiment was conducted in the form of a split-plot design with the main units, rats, arranged in a latin square with a two by two factorial arrangement of treatments in each row or column. The subunits were muscles with the tension measurements taken over time. This means that the muscle at time 1 hour, is actually a different subunit from a muscle measured at 2 hours.

Studentized Range/Maximum Gap Test

This statistical test also known as Murphy's Gap Test, was used in all cases to determine treatment differences where a significant difference was indicated. It utilizes decreasing values for significant difference and an altered method of declaring where significant difference exist. This results in a procedure in which a more sensitive exami-

nation of the results occurs while the accuracy seen in the least significant difference test is maintained. For a description of the actual procedure involved, see Murphy (1973).

CHAPTER IV

RESULTS AND DISCUSSION

Fiber Type Determination

In a preliminary study to select a muscle with which to study the effect of dantrolene, five muscle groups from each of five animals were examined with a light microscope. A random group of fibers at one location on each muscle section was observed and counted for red and white fiber types. A gross estimation of fiber type number was desired so intermediate fibers were subjectively placed in one or the other categories based on size and the amount of foramazen formed.

The mean values for percentage red fibers for each animal and muscle are shown in Table II. The muscle fibers ranged from 51.8% red fibers in the GA, to 45.9% red fibers in the GM to a low of 32.9% red fibers in the TB. Using the analysis of variance for a randomized complete block design, with the blocks being individual rats, and the treatments consisting of muscle location, it was determined that differences ($P < .05$) did exist among the muscles in percentage red fibers. Subsequent calculation of a least significant difference indicated that at the 5% level of probability, the GA contained a greater percentage red fibers than did

TABLE II

PERCENTAGE OF RED FIBERS IN RAT MUSCLE

Animal	GA[1]	GM	BF	RF	TB
		Percentage Red Fibers			
1	51.76	50.23	40.19	42.69	40.60
2	56.91	46.76	44.94	40.87	33.66
3	48.65	45.70	39.91	46.03	24.09
4	49.25	44.91	45.39	43.71	30.93
5	51.69	42.17	37.59	46.30	35.83
Mean Value	51.65*	45.94	41.60	43.92	33.02*

*Significant difference ($P < .05$)

[1]Gastrocnemius group (GA), Gluteus group (GM), and Biceps Femoris (BF), Rectus Femoris (RF), and Triceps Brachii (TB).

the remaining muscles. The GM, BF, and RF did not differ and the TB contained a significantly smaller percentage of red fibers. The GA was also easier to remove from the animal and possessed size and tensile properties favorable to the experiment.

Effect of the Injection

In two of the treatments, dantrolene injection and control injection, two variables were introduced - the injection and the addition of propylene glycol (PPG) into the animal. The injection was deemed essential to the experiment as a potential means of introducing DS into the animal in a practical manner. Propylene glycol was used because it readily dissolves DS and because it possesses a low level of toxicity for the rat.

Two questions need to be answered to interpret the data from the injected rats. They are (1) what effect did the added stress of the injection have on the animal? and (2) what effect did the PPG have on those characteristics being monitored?

The animals which were injected received a greater amount of handling prior to death than the others. In addition, injection of 2 ml solution via a 20 guage needle into the abdomen was required. This additional stress placed on the rats might be similar to that incurred by transporting and slaughtering animals in the meat industry. With additional stress, initial muscle tension and final

tension should be increased. In addition, initial pH should be lower due to the burst of activity accompanying the stress and final pH should be higher because of an initial decrease in glycogen content of the muscle following the stress. The stress should lower the concentration of ATP in the muscle at the time of death thus decreasing the time period needed to remove the remaining ATP resulting in an early onset of rigor mortis.

These effects have been anticipated through examining the exaggerated effects of stress on stress suseptible swine. In stress prone swine, activity invokes lactic acid acidosis with a concurrent drop in pH, and a quick stiffening of "rigor" formation (Topel, 1978). Price and Schweigert (1971) indicated that glycogen depletion in the animal is associated with the degree and duration of stress, and that the state of muscle glycogen influences the lactic acid concentration and final pH.

The second factor to be resolved was the effect of PPG on the characteristics under investigation. As mentioned earlier, Zaroslinski et al. (1971) recommended not using PPG intraperitoneally because of its muscle relaxant activities. The drug was introduced into the rat 1.5 hours prior to slaughter. Lehman and Newman (1937) indicated a maximum blood concentration occurred in mice from 30 minutes to two hours after administration of a two ml dose. It must be assumed, therefore, that in the injected rats the maximum level of the drug is present in the blood at death.

Bonnardeaux (1971) found that PPG was not only a relaxant at the central level as suggested by Zaroslinski, but also affected isolated smooth muscle.

Assuming that PPG acts as a relaxant, the muscle should exhibit the following effects: (1) PPG should result in a general inhibition of tension generation, and (2) PPG should result in a decrease in the rate of ATP breakdown causing an increase in time before rigor onset.

The effect of PPG on pH and lactate concentrations is difficult to determine. The addition of a muscle relaxant (curare) to pigs antemortem by McLoughlin (1970) resulted in a significantly higher pH immediately post mortem due to an inhibition of struggling and resultant energy depletion. Muscle relaxants should result in no major differences in pH over controls because they do not effect the amount of ATP metabolized by the muscle, but rather the rate of that metabolism. With PPG; however, it must be remembered that the drug itself is an energy source for muscle, and that the end point of PPG metabolism is pyruvate and lactic acid which accumulate in the cell lowering pH.

In each of the variable responses, including tension development across time, tension at rigor, time to rigor, lactate concentration, and gain, a significant source of variation was attributed to the factor ROW in the analysis of variance. The rows of each square were determined according to the weight of the rats used for each latin square with the heaviest rats placed on the top row and the lightest on the

bottom. Since the row mean weights were not identical between each square (Table III), a factor of pooled row among squares can be determined. Significance of this factor indicated that there was a difference in the variable response from row within and between squares. For example, row one of square one may have responded differently than row one of square two, or row one of square one may respond differently than row three of square one. The cause of variation is confounded between the animal size and the vertical position in the lab. Significance indicates that an experimental design was necessary which resulted in each treatment appearing only one in each row as was the case with the latin square design. A lack of significance indicated that a design in which the same treatment could have occurred any number of times in each row would have been adequate.

Effects of Dantrolene Sodium

Tension Development

A comparison of the four treatment effects on tension development is shown in Table IV and is displayed in Figure 5. No significant differences ($P < .05$) were indicated in tension development due to any treatment through the first three hours. Several trends do appear, however, which will be mentioned. The muscles from rats fed DS were consistantly lower in tension throughout the timed tests than the other three treatments. It was expected that the rats fed

TABLE III

A COMPARISON OF THE AVERAGE INITIAL WEIGHT BY ROW ACROSS THREE LATIN SQUARES

Row	Square 1 (g)	Square 2 (g)	Square 3 (g)
1	115.95	147.93	126.35
2	114.33	147.88	124.95
3	113.50	145.48	123.13
4	112.08	140.20	118.60

TABLE IV

THE INFLUENCE OF TIME ON RAT MUSCLE TENSION

Treatment	N	Hour 1	2	3	4
		(g)			
Control	12	5.75	6.13	7.49	8.61
Fed Dantrolene	12	4.89	5.89	7.08	8.13
Injection Control	12	6.14	7.69	10.03	11.46*
Injection Dantrolene	12	6.64	6.98	8.25	9.17

*Significant difference ($P < .05$)

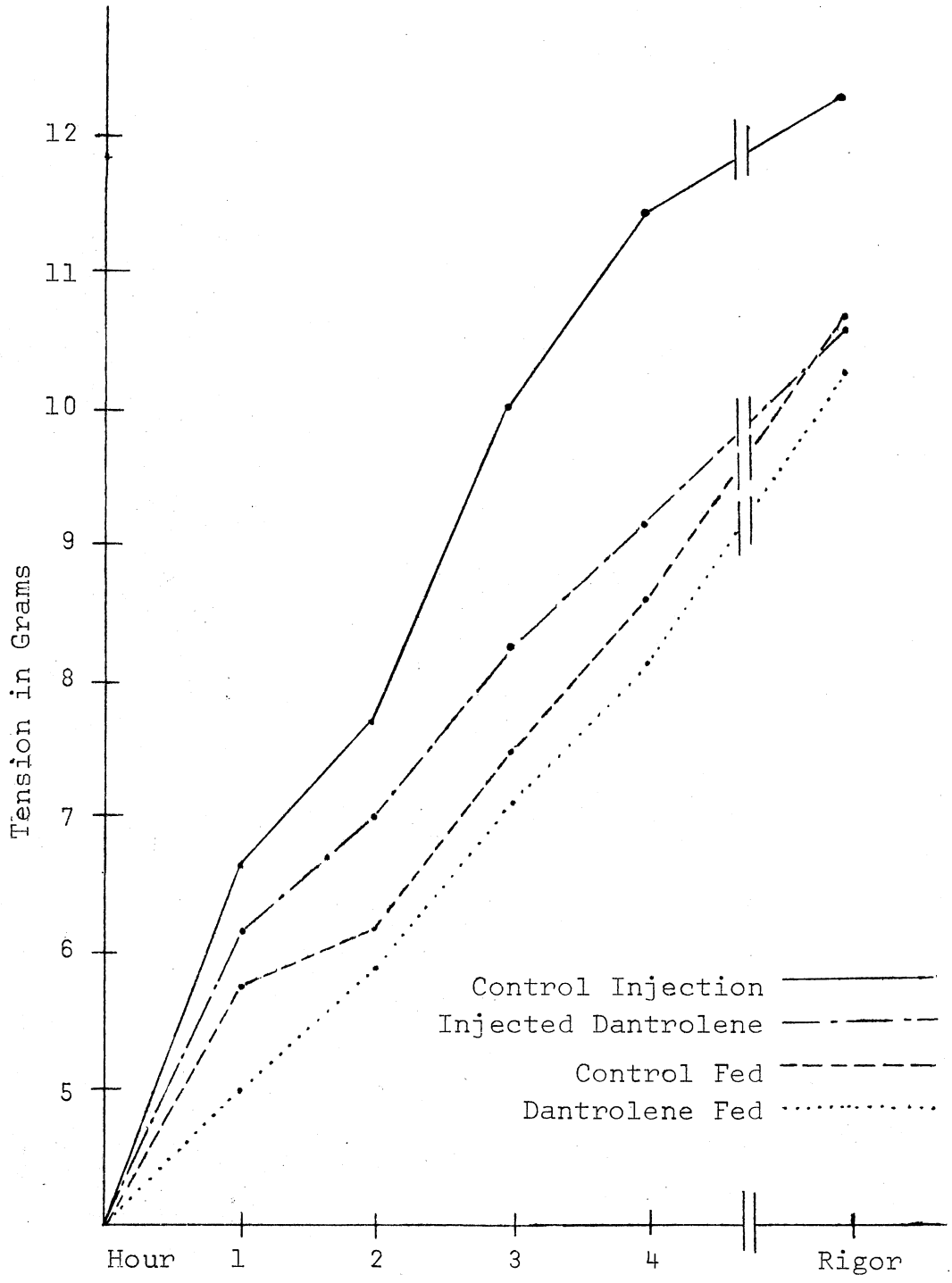


Figure 5. The Effect of Dantrolene Sodium and Time on Rat Muscle Tension

DS would not develop the tension that the control rats did as DS functions as a muscle relaxant.

In comparing rats injected with only carrier (PPG) to those injected with DS dissolved in carrier, a large increase in muscle tension was seen in those injected with PPG. It was anticipated that the extra handling and injection would show the effects of stress, but the DS effectively countered much of the additional tension. This indicates that if PPG is an active muscle relaxant, it is not nearly as powerful as DS. At hour four, the previous trends continued, but the muscles of the rats injected with PPG were significantly greater in tension than those from the other three treatment groups.

Tension at Rigor

The average tension of rat muscles at rigor is presented in Table V. The final tension of the rat muscles indicated that both treatment groups that received DS developed less final muscle tension than those which did not receive the drug, with the muscles from the rats injected with carrier increasing the most in tension. No significant difference ($P < .05$) occurred so the differences may be apparent rather than actual.

Time to Rigor Onset

The times required for the onset of rigor for each treatment are shown in Table VI. A difference ($P < .05$) was

TABLE V

TENSION OF RAT MUSCLE AT RIGOR ONSET

Treatment		Mean Tension (g)	Standard Deviation
Control	12	10.65	2.73
Fed Dantrolene	12	10.27	1.92
Control Injection	12	12.31	3.75
Injected Dantrolene	12	10.59	2.58

TABLE VI

TIME TO RIGOR ONSET IN RAT MUSCLE

Treatment	N	Mean Time to Rigor (min)	Standard Deviation
Control	12	347	70.6
Fed Dantrolene	12	352	74.7
Control Injection	12	298*	68.7
Injected Dantrolene	12	326	72.3

*Significant difference ($P < .05$)

seen between those rats which received a control injection of PPG and those on the other three treatments. Assuming the carrier PPG, is a muscle relaxant as indicated by Daroslinski et al. (1971) and Bonnardeaux (1971), the time delay before the onset of rigor should have increased rather than decreased, therefore, the actual decrease in time to rigor due to the injection stress alone could be greater than that which occurred. Dantrolene injected into the rat increased the time to rigor onset by approximately 30 minutes when compared to the muscles from those rats injected with carrier alone. This increase was expected because of DS relaxing effect resulting in lower energy consumption in the muscle. Some increase in time to rigor was also expected however, in the muscle receiving carrier if the PPG does in fact act as an extra strength source. Dantrolene fed to rats resulted in an average increase of five minutes to rigor onset over the control fed rats.

pH Measurement

The results seen in pH determination of the treatments are given in Table VII. A significant difference was seen between the rats receiving the DS injection and the other three treatments in average hydrogen ion concentrations. (The hydrogen ion concentrations were found by finding the tenth power of the pH measurement. These were then averaged and the mean pH was determined by finding the log of one over the hydrogen ion content.) It was expected that the

TABLE VII

pH VALUES OF RAT MUSCLE AT RIGOR ONSET

Treatment	N	Mean pH	Standard Deviation
Control	12	6.46	0.12
Fed Dantrolene	12	6.35	0.19
Control Injection	12	6.36	0.17
Injected Dantrolene	12	6.05	0.20

*Significant difference ($P < .05$)

rats fed DS would exhibit a lower pH due to the slight acidity of the drug, and that the pH of the PPG injected rats would reflect the increase in muscle substrate, but the reason for the large drop in pH seen in those rats injected with PPG and DS is not known. It is obvious, however, that there is more than a cumulative effect between the PPG and the DS.

Lactate Concentration

The average values for lactate concentration in the tissue supernatant fluid and in the muscle samples are shown in Table VIII. No significant differences due to treatment was demonstrated. The stress placed on the injected rats was expected to decrease the amount of glycogen in the muscle resulting in a decrease in the lactic acid content. The muscles from the groups receiving injections were not appreciably different from the control rats.

The procedure for lactic acid determination contained several steps increasing the chances for experimental error. Coupled with this was the inability to do duplicate tests on the muscle due to small muscle size.

A significant difference ($P < .05$) was seen between squares in lactate concentration. Examination of the data for each square showed a large increase in lactate concentration from square one to squares two and three. Although several trial tests were performed, it must be assumed that the results show improvement with practice.

TABLE VIII

LACTIC ACID CONCENTRATION IN TISSUE SUPERNATANT FLUID AND IN RAT MUSCLE

Treatment	N	Lactate Concentration		
		mole/ml sample	Standard Deviation	mole/g rat muscle
Control	12	3.11	.47	24.88
Fed Dantrolene	12	3.26	.52	26.08
Control Injection	12	3.09	.55	24.72
Injected Dantrolene	12	3.13	.36	25.04

Concentration of lactate per gram muscle tissue was found by multiplying the supernatant fluid concentration by eight (the number of milliliters of perchloric acid added per gram of tissue). Therefore the analysis is the same as that of the supernatant fluid concentration.

Rat Weight Gain

The results of the average weight gains for the treatments are given in Table IX. Murphy's Gap analysis divided the treatments into two distinct groups - those fed DS and those not fed DS. The rats which were fed DS showed noticeable discrimination against the DS treated veal. Since all groups were exposed to the Purina chow for identical periods of time, the intake of DS must have had a depressant effect on rat appetite. The similarity in gain among the other three groups was reassuring since the rats were supposedly treated in a similar manner.

TABLE IX

RAT WEIGHT GAIN DURING 30 DAYS ON FEED

Treatment	N	Mean Gain (g)	Standard Deviation
Control	12	68.95	10.8
Fed Dantrolene	12	57.42*	8.5
Control Injection	12	70.38	15.9
Injected Dantrolene	12	69.78	18.9

*Significant difference ($P < .05$)

CHAPTER V

CONCLUSIONS AND SUMMARY

Four treatments, (1) fed dantrolene sodium, (2) injected dantrolene sodium, (3) fed control, and (4) injected control, were applied to rats to examine the effects of dantrolene on the chemical and physical properties of muscle rigor mortis. The measurements taken on the rat gastrocnemius muscle included muscle tension at 1, 2, 3, 4 hours and at rigor onset, muscle pH at rigor, elapsed time to rigor onset, lactate concentration at rigor, and weight gain over the 30 day feeding period.

Rats receiving dantrolene sodium consistantly developed less tension than did their control counterparts. Rats receiving dantrolene also experienced rigor after a greater time had elapsed than in their controls. There was a significant difference in the case of the injected pair with control injected rat muscles entering rigor after 298 minutes whereas dantrolene injected rats took 326 minutes. Neither the feeding of dantrolene or the propylene glycol injection resulted in pH different from the control, however, a significantly lower value was obtained as a result of some interaction of the dantrolene and the propylene glycol in the dantrolene injection group. Lactate concentration

at rigor was not significantly different in any of the treatment groups.

No research prior to this has examined the effects of dantrolene sodium, a skeletal muscle relaxant, on post mortem rigor formation. Few significant differences have appeared although several encouraging trends are seen. In light of the effects on gain caused by the feeding of dantrolene, it is suggested that future research concentrate on injection of dantrolene as the method of introduction into the animal.

The effects seen in this experiment were caused by a level of dantrolene which was lower than that required to produce noticeable muscle relaxation in the rat. Higher dosage and/or an increased number of animals is recommended for further investigations. Further study should also include examinations of the relative toxicity of drug carriers, the metabolism of the carriers, and interaction effects with dantrolene such as that seen with pH at rigor.

In summary, further research is necessary to determine (1) whether or not dantrolene can cause significant tension inhibition in post mortem muscle, (2) whether or not tension reduction results in increased tenderness, (3) what residual compounds remain in dantrolene treated muscles, and (4) is there any practical application of rigor inhibition to the meat industry?

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A P P E N D I X

TABLE X

ANALYSIS OF VARIANCE FOR PERCENTAGE RED FIBERS IN SELECTED RAT MUSCLES

Source	DF	Sum of Squares	Mean Square	F
Total	24	1222.9804	-----	-----
Animals	4	76.2764	19.0691	1.3975
Muscles	4	928.3876	232.0969	17.0099
Residual Error	16	218.3164	13.6448	-----

TABLE XI

ANALYSIS OF VARIANCE FOR TENSION DEVELOPMENT BY HOUR

Source	DF	Sum of Squares	Mean Square	F
Total	191	1520.9083	7.9629	
Square	2	9.1002	4.5511	0.3118
Row	9	373.8555	71.0683	4.9375*
Position	3	20.2877	6.7625	0.4633
Sq. x Position	6	35.3503	5.8917	0.4036
Dantrolene	1	39.6942	39.6942	2.7195
Injection	1	97.6126	97.6126	6.6875*
Dant. x Injection	1	8.1263	8.1263	0.5567
Sq. x Dantrolene	2	1.3316	0.6658	0.0456
Sq. x Injection	2	62.5414	31.2707	2.1424
Sq. x Inj. x Dant.	2	10.5832	5.2916	0.3625
Error A	18	262.7309	14.5962	
Hour	3	391.4381	130.4794	97.6277*
Dantrolene x hour	3	6.1231	2.0410	1.5271
Injection x hour	3	14.3739	4.7913	3.5850*
Dant. x Inj. x Hour	3	8.3439	2.7831	2.0824
Sq. x Hour	6	3.0474	0.5079	0.3800
Row x Hour	27	70.7427	5.4015	4.0416
Position x Hour	9	9.9017	1.1002	0.8232
Sq. x Pos. x Hour	18	6.2134	0.3452	0.2583

TABLE XI (Continued)

Source	DF	Sum of Squares	Mean Square	F
Sq. x Dant. x Hour	6	3.0905	0.5111	0.3854
Sq. x Inj. x Hour	6	11.3491	1.8915	1.4153
Sq. x Dant. x Inj. x Hour	6	2.8930	0.4822	0.3608
Error B	54	72.1703	1.3365	

*Significant difference ($P < .05$)

TABLE XII

ANALYSIS OF VARIATION FOR TENSION

Source	DF	Sum of Squares	Mean Square	F
Total	47	380.3192	8.0919	1.3725
Square	2	2.7054	1.3527	0.2294
Row	9	168.2088	18.6899	3.1701*
Position	3	10.2542	3.4181	0.5798
Square x Position	6	12.5796	2.0966	0.3556
Dantrolene	1	13.2300	13.2300	2.2440
Injection	1	11.8008	11.8008	2.0016
Dant. x Injection	1	5.3333	5.3333	0.9046
Square x Dantrolene	2	6.1763	3.0881	0.5238
Square x Injection	2	34.7804	17.3902	2.9496
Sq. x Inj. x Dant.	2	9.1279	4.5640	0.7741
Residual	18	106.1225	5.8957	

*Significant difference ($P < .05$)

TABLE XIII

ANALYSIS OF VARIANCE FOR MINUTES TO RIGOR ONSET

Source	DF	Sum of Squares	Mean Square	F
Total	47	247095.917	5257.3599	
Square	2	25671.167	12835.5833	3.3222
Row	9	88455.250	9828.3611	2.5438*
Position	3	2276.917	758.9722	0.1964
Square x Position	6	19061.333	3176.8889	0.8223
Dantrolene	1	3234.083	3234.0833	0.8371
Injection	1	16800.083	16800.0833	4.3483*
Dant. x Injection	1	1518.750	1518.7500	0.3931
Square x Dantrolene	2	17967.167	8983.5833	2.3252
Square x Injection	2	638.167	319.0833	0.0826
Sq. x Inj. x Dant.	2	1928.000	964.0000	0.2495
Residual	18	69545.000	3863.6111	

*Significant difference ($P < .05$)

TABLE XIV

ANALYSIS OF VARIANCE FOR pH AT RIGOR ONSET

Source	DF	Sum of Squares	Mean Square	F
Total	47	2.3608	0.0502	
Square	2	0.1128	0.0564	2.5520
Row	9	0.2789	0.0310	1.4027
Position	3	0.1895	0.0632	2.8597
Square x Position	6	0.2132	0.0355	1.6063
Dantrolene	1	0.4505	0.4505	20.3846*
Injection	1	0.4313	0.4313	19.5158*
Dant. x Injection	1	0.1576	0.1575	7.1267*
Square x Dantrolene	2	0.0074	0.0037	0.1674
Square x Injection	2	0.0953	0.0477	2.1584
Sq. x Inj.	2	0.0270	0.0135	0.6108
Residual	18	0.3974	0.0221	

*Significant difference ($P < .05$)

TABLE XV

ANALYSIS OF VARIANCE FOR LACTATE CONCENTRATION AT RIGOR ONSET

Source	DF	Sum of Squares	Mean Square	F
Total	47	10.3652	0.2205	
Square	2	4.4429	2.2215	22.1486*
Row	9	2.3759	0.2640	2.6321
Position	3	0.3683	0.1228	1.2243
Square x Position	6	0.4098	0.0683	0.6810
Dantrolene	1	0.1217	0.1217	1.2134
Injection	1	0.0667	0.0667	0.6650
Dant. x Injection	1	0.0362	0.0362	0.3609
Square x Dantrolene	2	0.2729	0.1364	1.3599
Square x Injection	2	0.2875	0.1438	1.4337
Sq. x Inj. x Dant.	2	0.1782	0.0891	0.8883
Residual	18	1.8050	0.1003	

*Significant difference ($P < .05$)

TABLE XVI

ANALYSIS OF VARIANCE FOR RAT WEIGHT GAIN

Source	DF	Sum of Squares	Mean Square	F
Total	47	10809.4267	229.9878	
Square	2	3470.8504	1735.4252	25.7863*
Row	9	2732.7413	303.6379	4.5117*
Position	3	11.3783	3.7928	0.0564
Square x Position	6	719.7829	119.9638	1.7825
Dantrolene	1	441.6533	441.6533	6.5624*
Injection	1	571.3200	571.3200	8.4891*
Dant. x Injection	1	358.6133	358.6133	5.3285*
Square x Dantrolene	2	89.1204	44.5602	0.6621
Square x Injection	2	755.4463	377.7231	5.6125*
Sq. x Inj. x Dant.	2	447.1129	223.5564	3.3218
Residual	18	1211.4075	67.3004	

*Significant difference ($P < .05$)

VITA

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