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THE EFFECT OF SELECTED ELECTRICAL STIMULATION INTENSITIES ON THE GLYCOGEN DEPLETION PATTERN IN FAST AND SLOW MOUSE MUSCLE

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THESIS

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ABSTRACT

The extensor digitorum longus (EDL) and soleus (SOL) muscles of forty mice were subjected to direct electrical stimulation at intensities representing 10, 30, 50, 70 and 100% of maximal tetanic tension. The glycogen depletion pattern in the stimulated muscles was evaluated qualitatively by the PAS stain. Non-stimulated, sham-operated muscles of the contralateral leg served as the controls.

As stimulation voltage was increased, there was an overall reduction in the PAS staining intensity in both EDL and SOL muscles. Electrical stimulation intensities in the 10 and 30% groups produced significantly more glycogen depleted fibers in the SO fiber type than FOG in SOL. The depletion pattern in EDL, at these lower voltages, was inconclusive.

At stimulation intensities between 30 and 70% of maximal tetanic tension, a significant trend towards more glycogen depleted FG fibers was observed in EDL compared to FOG fibers. This marked a predominant shift to the glycolytic fiber pool in terms of recruitment of muscle fibers at higher intensities of stimulation. In SOL, however, neither the SO nor the FOG fiber type displayed a specific recruitment pattern due most likely to the absence of the FG fiber in this muscle.

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CHAPTER I

THE PROBLEM

Introduction

Skeletal muscle fibers have been classified histochemically into distinct fiber types characterized by differences in contractile, metabolic and functional parameters (Close, 1972). The histochemical mosaic in a given muscle can undergo considerable change with various manipulations such as nerve cross-union, exercise, electrical stimulation, surgically induced overload and during the course of normal maturation and development (Burke and Tsairis, 1974).

The determination of glycogen levels in skeletal muscle by biochemical and histochemical means before and after bouts of exercise has suggested that muscle fibers may be used differentially depending on the nature of the work performed by the muscle (Armstrong et al., 1974; Baldwin et al., 1973, 1975). Stein and Padykula (1962) suggested that variations in glycogen content, as reflected by the Periodic-Acid Schiff (PAS) staining intensity, illustrated various phases in a cycle of glycogen anabolism and catabolism. They were able to show that the PAS stain correlated highly ($r = 0.87$) with a quantitative glycogen assay and used it to follow glycogen depletion in muscle fibers as an indicator of muscle fiber recruitment.

Experimentation with animals has demonstrated that differential rates of glycogen depletion occur in the fibers of skeletal muscle during electrical stimulation and after varying periods of running on a rodent treadmill. In electrical stimulation studies conducted by Kugelburg and Edstrom (1968) involving individual nerve fibers, the entire nerve and muscle, a preferential loss of glycogen was found in the fast twitch glycolytic fibers (FG). Armstrong et al., (1974; 1975) and Gillespie et al., (1974) showed that oxidative fiber groups were recruited at low to moderate exercise intensities and that the glycolytic fibers were activated at the higher intensities.

Rationale

Many studies have been conducted using indirect stimulation techniques, but at present little information exists concerning the role of the different muscle fiber types during direct electrical stimulation. If it could be shown that a selective recruitment of muscle fibers could be achieved through direct electrical stimulation, it would serve as a useful model for muscle physiologists interested in studying the metabolic characteristics of the different fiber types in skeletal muscle.

Statement of the Problem

The purpose of this investigation was to examine the effect of selected electrical stimulation intensities representing 10, 30, 50, 70 and 100% of maximal tetanic tension

on the glycogen depletion pattern in the fast extensor digitorum longus and slow soleus muscles of the mouse.

Limitations

This study is limited to the examination of two muscles: fast extensor digitorum longus (EDL) and slow soleus (SOL) muscles of the mouse, and therefore, does not represent all muscles of the mouse.

The range of direct stimulation intensities was restricted to five voltages representing 10, 30, 50, 70 and 100% of maximal tetanic tension.

The PAS ratings of the photomicrographs were rated by one evaluator.

Definition of Terms

Maximal tetanic tension: the peak tension produced in the muscle at maximum voltage at a frequency of 100Hz.

PAS Staining Intensities: individual muscle fibers were rated as negative, light, moderate or dark according to the reference photomicrograph.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction

This chapter outlines the review of the related literature under the following headings: classification of skeletal muscle fibers; glycogen as a fuel for muscular contraction; the PAS stain for glycogen; electrical stimulation of muscle; and the effect of electrical stimulation on glycogen in fiber types.

Classification of Skeletal Muscle Fibers

The criteria for classification of skeletal muscle fibers into distinct "types" has been based on electron-microscopical, physiological, biochemical and histochemical studies. Close (1972) demonstrated a variety of fiber nomenclatures in animal skeletal muscles based on different histochemical procedures. Close employed a three fiber system: red, white and intermediate.

Peter et al, (1972) adopted a three fiber classification scheme based on the histochemical staining response to a myofibrillar ATPase stain and an oxidative enzyme stain (NADH-TR). On the basis of the colour intensity of the ATPase stain (pH 9.4), a differentiation between slow twitch and fast twitch fibers was made. An alkaline pre-incubation resulted in the slow twitch fibers being light staining and

the fast twitch fibers as dark staining. An acid pre-incubation produced a reversal in staining intensity which was commonly used as a check in histochemical procedures. The oxidative enzyme stain, NADH-TR, was used to separate the fast twitch fibers into a high oxidative and a low oxidative category. Peter and his co-workers designed a nomenclature system that reflected the metabolic and contractile characteristics of the different fiber types. He classified the slow twitch fibers as SO, indicative of their slow contracting and oxidative properties, and subdivided the fast twitch fibers into FG and FOG. The FG were fast twitching fibers with high glycolytic capacities, but low in oxidative capacity. The FOG fibers, however, were fast contracting fibers, yet had high glycolytic and oxidative properties.

The classification of human muscle fiber types as type I or II was adopted by Engel in 1974. This two fiber system was based on the histochemical ATPase (pH 9.4) following an alkaline pre-incubation. Type I fibers were slow contracting and type II were fast contracting fibers. Other investigators, Gollnick et al., 1972; and Costill et al., 1976; prefered to use the terms ST for the slow contracting and FT for the fast contracting fibers.

Brooke and Kaiser (1970) demonstrated that the ATPase activity of the fibers in human muscle was greatly influenced by not only pre-incubation pH, but also by temperature and duration of the pre-incubation. They proposed a four fiber system, using the type I, II classification,

subdividing the type II fibers into three sub-categories (IIa, IIb and IIc). The type IIa and IIb correspond to the FOG and FG fiber respectively (Brooke and Kaiser, 1974). The IIc fiber has been commonly referred to as the undifferentiated fiber (Brooke and Kaiser, 1974) which is prevalent in developing muscle and rarely seen in mature muscle. Dubowitz and Brooke (1973) showed that the four fiber system, based on pH lability of the ATPase stain, was identifiable in other animal species.

Glycogen as a Fuel for Muscular Contraction

Muscle glycogen is a substrate which serves as an important energy source for muscular contraction. Glycogen and the two major enzymes involved in its regulation, glycogen synthetase and phosphorylase, have been reported to vary between the fiber types in the skeletal muscle of several species including man (Stein and Padykula, 1962; Kugelburg and Edstrom, 1968; Gillespie et al., 1970; and Peter et al., 1972). Assuming that glycogen synthetase and phosphorylase activity reflect the ability of the muscle fiber to synthesize and utilize glycogen respectively, it can be concluded that fibers with high ratios of the previously mentioned enzymes would predominantly use glycogen as their source of energy. Engel (1974), Stein and Padykula (1962) and Dubowitz and Pearse (1960) have confirmed that the type II fiber is rich in glycogen and has high enzyme activities for glycogen synthetase and phosphorylase.

Glycogen synthetase is the rate-limiting enzyme in glycogen synthesis (Lehninger, 1975). This enzyme exists in two forms, the "D" and "I" form. The "D" form is dependent on glucose-6-phosphate and the "I" form is independent of glucose -6- phosphate. The two forms are interconvertible by mechanisms involving phosphorylation of the "I" form and dephosphorylation of the "D" form. Hultman et al., (1971) and Danforth and Lyon (1964) demonstrated that the activity of the "I" form was inversely related to the glycogen content of the muscle fiber. Under most physiological conditions, conversion to the "I" form of glycogen synthetase seems to be the most important factor involved in glycogen synthesis occurring in skeletal muscle (Saltin and Pernow, 1970). The regulation of glycogen synthesis and breakdown is illustrated in Figure 1.

Glycogen is hydrolyzed to glucose -1- phosphate by the enzyme phosphorylase. This enzyme also exists in two forms: the inactive "b" form and the active "a" form. Phosphorylase "b" may be activated to the "a" form by the activation of adenylcyclase which in turn activates a protein kinase. The "a" form can be converted back to the "b" form by the enzyme phosphorylase phosphatase. Danforth et al., (1962) showed nearly a complete conversion of phosphorylase "b" to the "a" form in electrically stimulated mouse muscle in as short a time as three seconds.

The PAS Stain

The Periodic Acid-Schiff (PAS) stain has been widely

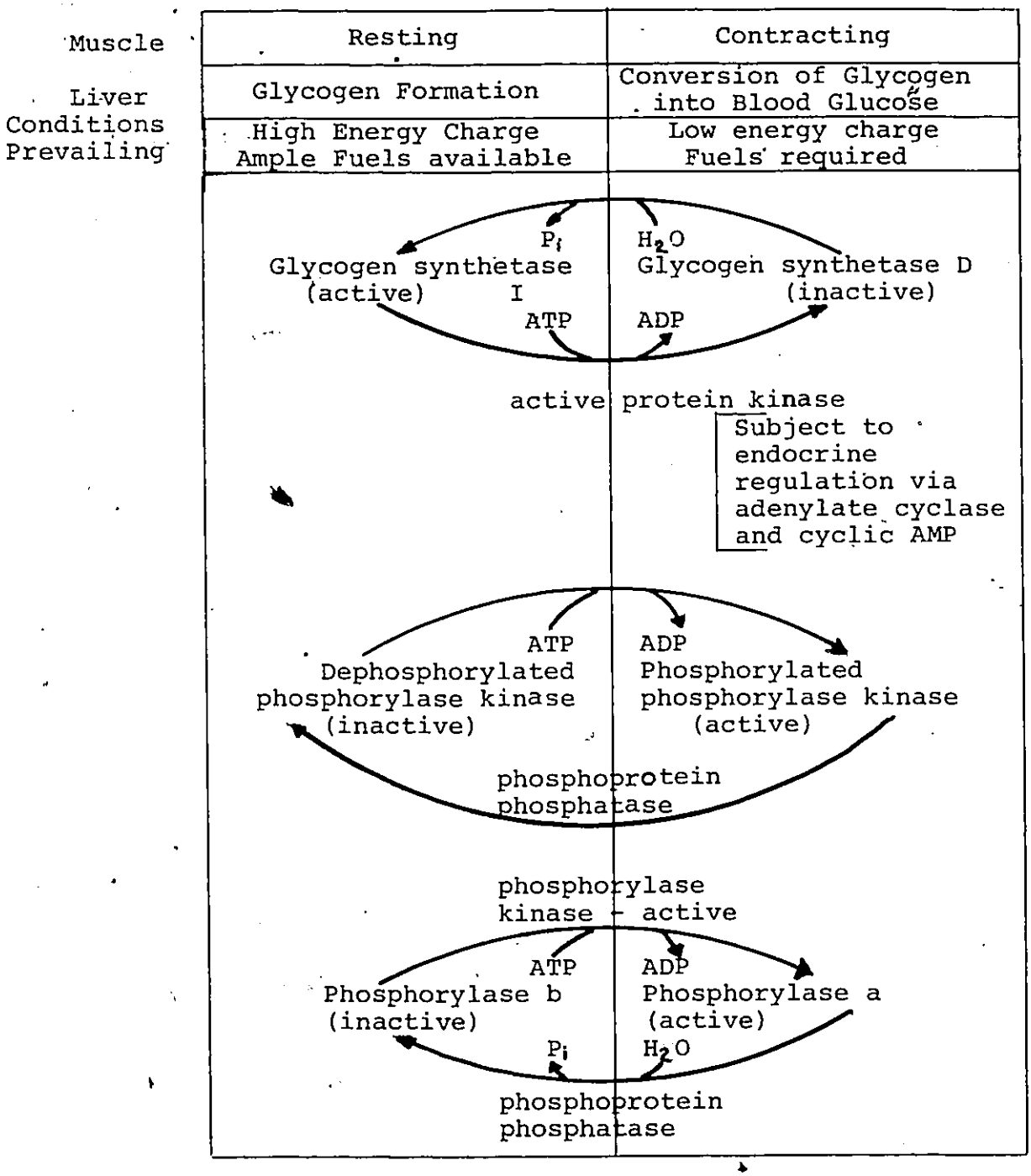

Figure 1: - Summary of the Regulation of Glycogen Synthesis and Breakdown of Mammalian Liver and Muscle.

Lehninger, 1975 .

used in histochemistry to qualitatively measure glycogen as well as other polysaccharides, neutral polysaccharides, muco-and glycoproteins and glycolipids (Pearse, 1961). Periodic acid acts as the oxidizing agent and breaks the "C-C" bonds occurring in the 1:2 glycol groups producing dialdehydes. The aldehydes then combine with the Schiff's Reagent to produce a reddish product which varies in intensity of colour according to the number of aldehyde groups produced and the type and amount of polysaccharide present (Pearse, 1961).

The depletion of muscle glycogen, demonstrated histochemically by the PAS stain, has served as an indicator of muscle fiber recruitment in exercise and electrical stimulation studies (Edgerton et al., 1972; Burke et al., 1971). The reliability of the PAS stain as a technique for the qualitative analysis of glycogen levels has been demonstrated by Stein and Padykula (1962). They were able to show that the PAS stain correlated highly (r=0.87) with a quantitative glycogen assay. Edgerton (1970), as well found a similar correlation for glycogen measured in homogenates of stimulated muscles with the percentage of PAS-positive fibers in the same muscles.

The PAS stain, however, has several limitations. Due to the subjective nature of fiber ratings, objectivity and reliability coefficients of the rating procedure should be established. It is also possible that resynthesis of glycogen within the muscle fiber may occur, which may mask

the true depletion pattern. However, Hultman et al., (1971) reported that rates of glycogen resynthesis are slow in comparison to glycogenolysis. The last and most critical limitation of the PAS stain is that the staining intensity appears to be at a maximum at glycogen levels exceeding 80 mmol. glucose units x kg^{-1}. Therefore, changes in glycogen content above this level cannot be detected as changes in PAS staining intensity, thereby making misleading results possible (Gollnick et al., 1972).

Despite the limitations of the PAS stain, it has been widely used in exercise and motor unit recruitment studies to show the fatiguability of the different muscle fiber types.

Electrical Stimulation of Muscle

Under natural conditions, the muscle fiber action potential is produced in response to transmitter release at the motor end-plate. It is also possible, however, to directly stimulate the muscle fiber through electrodes to mimic the events which occur at the post-synaptic site.

The electrical stimulus is employed to change the muscle fiber membrane potential to a critical value so as to allow the inflow of sodium ions into the cell. As a result, a lateral redistribution of charge occurs and a current flows out from the depolarized point (Katz, 1966). This current flow depolarizes nearby regions of the membrane and by local current flow, other regions of the membrane are activated. This spread of the action potential down the

muscle fiber is known as cable conduction in contrast to saltatory conduction seen in myelinated nerve fibers.

Excitation-contraction coupling is the sequence of events which links the electrical activity of the muscle fiber membrane to the activation of the myofibrils and the development of tension. Sandow (1970) describes the coupling phenomenon as a sequence of interlinked stages: (1) coupling begins with the depolarization of the muscle fiber membrane beyond the critical threshold value; (2) followed by a transverse spread of electrical activity into the interior of the fiber; (3) the internal depolarization activates the sarcoplasmic reticulum, leading to a release of calcium ions; and (4) calcium ions diffuse to the contractile apparatus where interaction with the myofilaments, the initiation of cross-bridge formation and generation of tension occurs.

The amount of tension that a muscle fiber can develop per unit cross-sectional area is dependent upon the volume of contractile machinery in relation to the total fiber volume and the mode of arrangement of the myofilaments within the contractile component (Huddart, 1975). The tension in electrically stimulated muscle may be varied by the voltage applied to the muscle and the frequency of discharge of the electrical pulses.

Studies conducted by Sandow (1970) demonstrated that increasing voltage of stimulation increases the proportion of muscle fibers that are excited. Voltages of a supra-maximal nature were used in studies by Edgerton et al., (1970)

and Kugelburg and Edstrom (1968) which caused the fast glycolytic fibers to fatigue rapidly.

Kjellmer (1964) demonstrated that contractions would not be affected by ischemia as maximal vasodilation is reached at a frequency of 5/sec in skeletal muscle and the flow of blood is still high at a frequency of 10/sec. Kugelburg and Edstrom (1968) noted that fibers in rat muscle fatigued earlier and was more marked when the frequency of stimulation was increased from 5 to 10/sec.

The amount of sarcoplasmic reticulum (SR) and the development of the transverse tubular system (TTS) within fast and slow skeletal muscle has been shown to be different. Luff and Atwood (1971) and Shafiq et al., (1969) showed that in adult muscles, the SOL fibers contained significantly less SR and TTS than EDL in the mouse. Falk and Fatt (1964) attributed the relatively high values for membrane capacitance of skeletal muscle fibers to the TTS. Luff and Atwood (1971) measured the total membrane capacitance in mouse EDL and SOL muscle fibers and found a mean of 5.3 uF/cm^2 for EDL fibers compared to 3.1 uF/cm^2 for SOL fibers. They concluded that due to the difference in surface area of the TTS and SR, the muscle fibers of EDL and SOL have different membrane electrical properties.

Furthermore, Pease et al., (1965) compared the calcium uptake in vitro in EDL and SOL muscle and concluded that more calcium seemed to accumulate in the fibers of EDL than those in SOL.

The Effect of Electrical Stimulation on Glycogen in Fiber Types

Relatively few studies have investigated glycogen depletion patterns in electrically stimulated muscle. It is apparent, however, that direct or indirect electrical stimulation using supra-maximal voltages selectively depletes glycogen in the FG fiber. Edgerton et al., (1970) and Kugelburg and Edstrom (1968) demonstrated that glycogen depletion measured by the PAS stain occurred predominantly in the FG fibers in guinea pigs and rats respectively. In the medial gastrocnemius of guinea pigs, Edgerton classified 97% of the FG fibers as PAS negative after one hour of indirect stimulation to the sciatic nerve at a frequency of 5/sec. As well, stimulation was applied in a direct manner and the authors noted identical results concluding that the neuromuscular junction did not play a role in the specific fiber recruitment during electrical stimulation.

The study by Kugelburg and Edstrom (1968) utilized the muscles of anterior tibialis and soleus of the rat. By varying supra-maximal stimulation duration from 30 seconds to two hours, they were able to demonstrate different patterns of glycogen depletion. At a frequency of 5/sec, greater than 90% of the FG fibers were PAS negative in anterior tibialis at the end of 5 minutes of stimulation. The FOG fiber showed less of a depletion effect, with very few fibers classified as negative. The SO fibers in both muscles showed very little glycogen depletion as a result of

the stimulation indicating that very little glycogen was metabolized under the experimental conditions.

Burke and his co-workers (1973) investigated the relationship between the physiological properties of stimulated single motor units of cat gastrocnemius muscle and the histochemical and morphological properties of the muscle fibers making up the same units. They classified the majority of muscle units into three categories: FF (fast contracting, fatigue sensitive; FR (fast contracting, fatigue resistant; and S (slow contracting, very fatigue resistant). Their work also demonstrated that the muscle units within each physiological type had the same histochemical profile.

The metabolic differences between the three fiber types may account for the preferential use of one fiber type during electrical stimulation. The FG fiber has a high capacity for anaerobic glycolysis whereas the FOG and SO fibers depend less on anaerobic glycolysis and more on oxidative glycolytic pathways for ATP (Dubowitz and Pearse, 1960). Edgerton et al., (1970) believed that glycogen was spared in the SO and FOG fibers as a result of their capacity for producing energy by oxidative means.

On the other hand, the recruitment of the FG fiber may indicate that muscle fibers have different thresholds for activation. Recent research by Campion (1974) showed that the different fiber types of guinea pig muscle have different resting membrane potentials (RMP) as well as potassium and sodium concentrations. Harris and Luff (1970)

demonstrated that in mouse muscle fibers, the RMP appeared to be a function of the age of the animal. In younger animals, the RMP in slow muscle was higher than in fast muscle. In mature animals, the reverse was true indicating that RMP is directly related to the age of the animal. They also noted that slow muscles reached electrophysiological maturity at an earlier age than did fast muscles.

Summary

Distinct muscle fiber types have been shown to have different metabolic and contractile characteristics. Glycogen depletion, demonstrated histochemically by the PAS stain, has been a useful marker to identify if a muscle fiber had been contracting. The histochemical changes in the PAS stain in exercise and electrical stimulation studies suggest that the metabolic nature of the different fiber types may play a role in the recruitment of specific fibers and the development of muscular fatigue. The development of an electrical stimulation model which would selectively recruit a particular fiber type would serve to greatly increase our knowledge of the properties of the various fiber types in skeletal muscle.

CHAPTER III

METHODOLOGY

Introduction

This chapter outlines the methodology of the study under the headings of sample selection, experimental protocol, histochemical procedures, photographic techniques and statistical design.

Sample Selection

Forty male black C57 mice obtained from Bio Breeding Laboratories of Canada Limited ranging in age from twelve to sixteen weeks were used in this study. All animals were housed in the Animal Care Services within the Faculty of Medicine at the University of Ottawa. Food and water were made available ad libitum. Eight mice were randomly assigned to each of the five experimental stimulation groups corresponding to 10, 30, 50, 70 and 100% of maximal tetanic tension. Within each experimental group, four mice were assigned to the EDL group and the remaining four comprised the SOL group. Table 1 outlines the assignment of animals to the various treatment groups.

Experimental Protocol

In this study, muscles EDL and SOL of the mouse were directly stimulated at selected stimulation intensities using

Table 1

Experimental Group Classifications

Animal Number	Stimulation Group	Muscle
1-4	10%	EDL
5-8	30%	EDL
9-12	50%	EDL
13-16	70%	EDL
17-20	100%	EDL
21-24	10%	SOL
25-28	30%	SOL
29-32	50%	SOL
33-36	70%	SOL
37-40	100%	SOL

a platinum-plated stimulating electrode connected to a Grass SD 9 Stimulator. The transducer was connected to a Grass Model 7 Polygraph for the graphic recording of isometric tension from the stimulated muscle. The polygraph was calibrated before each experiment as outlined in Appendix A.

The mouse was anesthetized with sodium pentobarbital (60 mg/ml.) administered intraperitoneally, followed by smaller doses as necessary. The hindlimbs were shaved and the animal was immobilized in a supine position on a dissecting board. In each leg, the experimental muscle was surgically isolated, with care taken to avoid disruption of the nerve and blood supply. A length of surgical thread was attached to the distal tendon and the tendon was cut. The thread was then attached to the transducer. The contralateral muscle was used as the control and was not stimulated, but was surgically isolated and stretched to the same tension as the

experimental muscle. A pin was inserted through the patella to immobilize the lower limb.

The stimulating electrode was placed on either side of the belly of the muscle and the muscle was stretched to a length which produced maximal twitch tension. Both experimental and control muscles were bathed in a mouse Ringers solution as outlined in Appendix B.

With the muscle at optimal length, it was directly stimulated at one pulse every five seconds at a duration of one millisecond. The voltage was then stepwise increased until maximum twitch tension was recorded. At maximum voltage, the frequency was increased to 100 H_z and a brief tetanus was administered and the tension recorded. The voltage was then adjusted so as to yield the required percentage of maximal tetanic tension for the duration of the experiment. Table 2 outlines the stimulation parameters used in the study. The muscle was rested for two minutes then repeatedly stimulated for 15 seconds and rested for 45 seconds. This regimen was continued for a period of one hour.

Table 2

Experimental Stimulation Protocol

Muscle	Frequency	Duration	Stim./Rest
EDL	40 Hz	1 msec	15/45
SOL	25 Hz	1 msec	15/45

Histochemical Procedures

Upon completion of the experiment, the muscles from the experimental and control leg were excised and weighed on a precision torque balance. The muscle was then embedded in a piece of fresh liver to assure proper orientation and rapidly frozen in isopentane chilled in liquid nitrogen. The muscles were stored in pre-chilled containers at -60°C until histochemical analysis was performed.

Six serial sections per muscle were cut at a thickness of 10u in a cryostat at -20°C. The sections were mounted on coverslips and left to air dry for a period of fifteen minutes. The histochemical analysis consisted of a myofibrillar ATPase stain according to the method of Padykula and Herman (1955) modified by a pre-incubation at pH 10.4 as described by Guth and Samaha (1969) and Khan et al., (1974); reduced nicotinamide adenine dinucleotide diaphorase-tetrazolium reductase (NADH-TR) oxidative enzyme stain according to the method of Novikoff et al., (1961) and the periodic Acid-Schiff (PAS) reaction for glycogen content (Pearse, 1961).

Photographic Techniques

Photomicrographs of the serial sections mounted on microscope slides were taken by a Canon F-1 camera through a microscope adapter. Light intensity and shutter speed were maintained identical for each picture.

Black and white prints were processed for the

subjective ratings of the muscle fibers as PAS negative, light,moderate or dark according to the method used by Kugelburg and Edstrom (1968) and other investigators (Gollnick et al., 1972).

Statistical Design

Reliability in the rating of muscle fibers for fiber type and PAS staining intensity was established by a test-retest procedure. A set of three photomicrographs, chosen at random from each muscle group, was used to establish reliability. Muscle fibers were rated as either slow oxidative (SO), fast oxidative-glycolytic (FOG) or fast glycolytic (FG) using the myosin ATPase and NADH-TR stains; and within each of these classifications, each fiber was rated as negative (N), light (L), moderate (M) or dark (D) with the PAS stain.

The Chi Square test was applied to determine if the mean total number of muscle fibers was significantly different across the ten treatment groups for each of the two muscles.

To test for homogeneity of muscle fiber types, a Chi Square test was applied to determine if there exists a significant difference between the groups when frequency counts were expressed as proportions for each fiber type compared to the total number of fibers.

Since the effect of increasing electrical stimulation intensity would decrease the glycogen content of the muscle fibers, a statistical test was not applied between the

experimental and control muscles because it would be assumed that there would be a significant difference.

To determine if there was a significant difference in the glycogen depletion pattern in the experimental muscles between the two fiber types, a Chi Square test for independence was applied in which a correction was made for unequal sample sizes according to the method of Snedecor (1956) which involves changing frequencies into percentages.

CHAPTER IV

RESULTS

Introduction

The purpose of this study was to investigate the effect of selected electrical stimulation intensities representing 10, 30, 50, 70 and 100% of maximal tetanic tension on the glycogen depletion pattern in fast EDL and slow SOL muscles of the mouse. The results are presented as follows: reliability; analysis of total fibers rated per group for each muscle; homogeneity of EDL and SOL samples for muscle fiber type; PAS staining intensities of the muscle fiber types in EDL and SOL expressed as mean percentages for each group; and Chi Square analysis of independence of fiber type in EDL and SOL according to the method of Snedecor (1956) using percentages.

Reliability

The results of the test for reliability are presented in Tables 3 and 4.

The Chi Square test results for reliability indicate that the method of rating of the serial photomicrographs had a high degree of reliability as the largest X^2 value of 4.868 is far below the critical X^2 value of 7.815. Hence, no significant sampling bias was observed.

Table 3

Test-Retest Data for Reliability in Classification of PAS Staining Intensities of Muscle Fibers

Treatment	Fiber Type	PAS Staining Intensity N	L	M	D
Test (EDL)	FOG	0	4	58	12
	FG	0	15	44	21
Retest	FOG	0	6	61	9
	FG	0	13	52	18
Test (SOL)	SO	0	22	86	2
	FOG	0	14	90	7
Retest	SO	0	24	82	5
	FOG	0	21	85	6

* Random selection resulted in control muscle 4 of the 50% treatment group of EDL and experimental muscle 4 of the 30% treatment group of SOL being used here to establish reliability.

Table 4

Chi Square Test of Reliability Data

Fiber Type		X^2 Test-Retest	Probability Level
EDL:	FOG	1.905	.59
	FG	2.140	.53
SOL:	SO	4.868	.195
	FOG	3.910	.275

* critical value of $X^2 = 7.815$ with df = 3, α .05

Number of Muscle Fibers Rated

Chi Square analysis indicated that there were significant differences in the total number of fibers rated across the ten groups in both EDL and SOL muscles. In the EDL group, six out of ten groups were significantly different whereas in the SOL group, five out of ten were significantly different (see Appendix C).

Homogeneity of Fiber Types

The results of the Chi Square test for homogeneity of muscle fiber type proportions, expressed as frequency counts, are presented in Appendix D. The test demonstrated that there was no significant difference in muscle fiber type proportions across all groups.

PAS Staining Intensities of Fiber Types

Frequency data of mouse EDL and SOL muscle fibers rated with the PAS stain is presented in Appendix E. The histochemical glycogen depletion pattern of the forty muscles in each of the EDL and SOL groups subjected to electrical stimulation, expressed as a percentage of the mean fiber population, is illustrated in Figures 2 and 3 respectively.

Fiber Type Independence in EDL and SOL

Due to the fact that the number of fibers counted was significantly different but the proportions of fiber types were not, frequency counts were converted to percentages

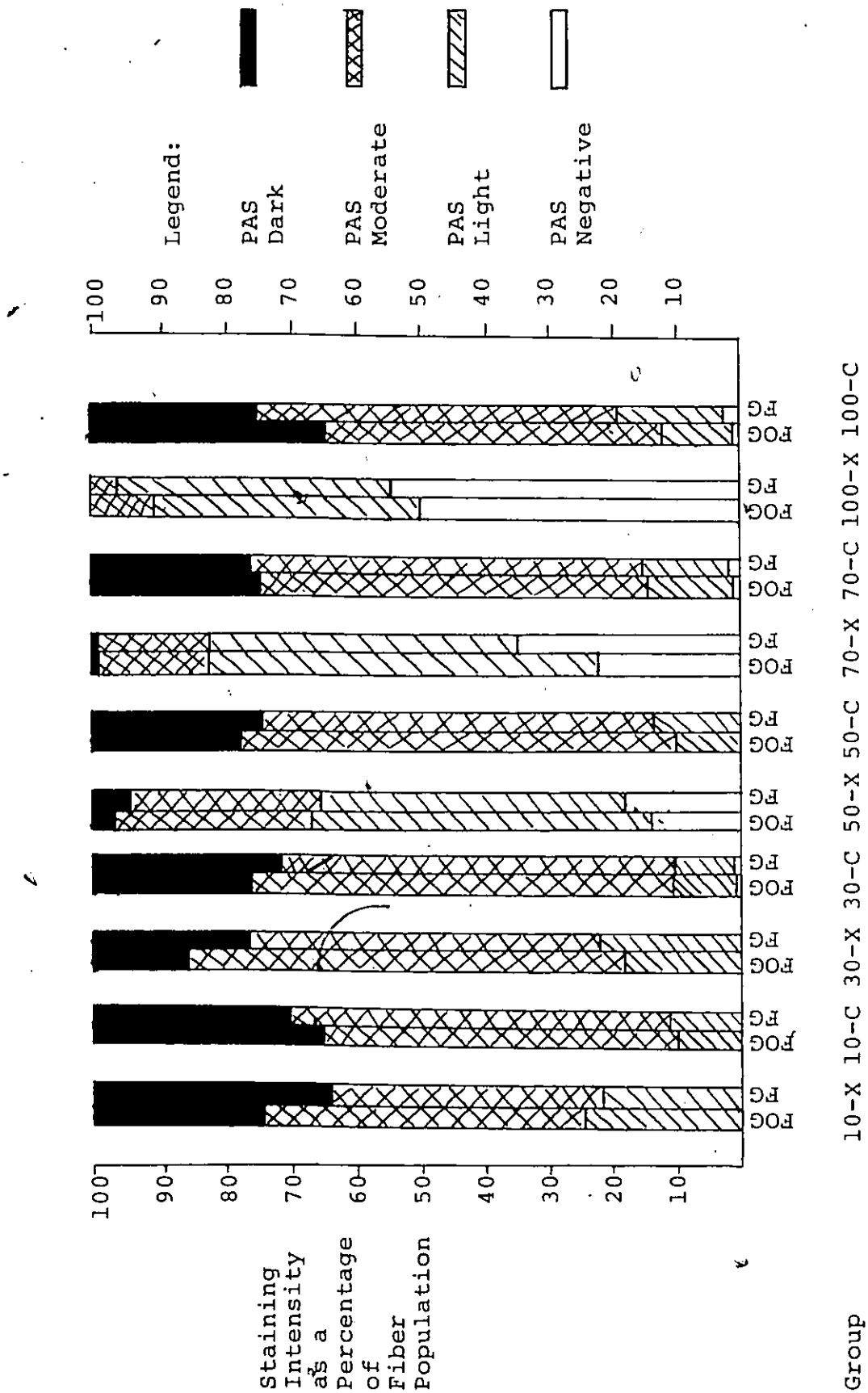

Figure 2: - Mean Ratings of PAS Staining Intensity of Mouse EDL Muscle Fibers Expressed as a Percentage of the Mean Fiber Population for Each Treatment Group.

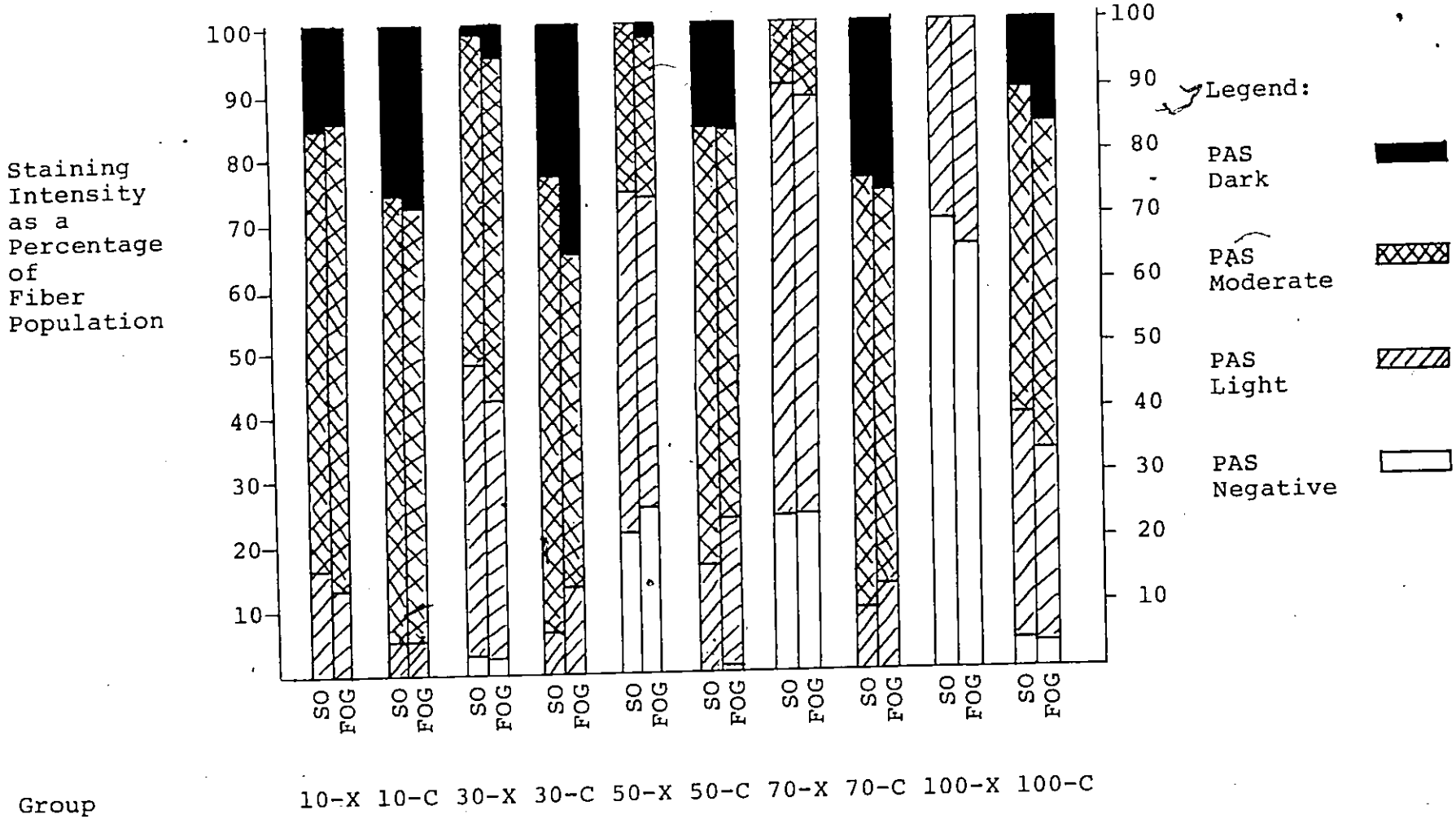

Figure 3: - Mean Ratings of PAS Staining Intensity of Mouse SOL Muscle Fibers Expressed as a Percentage of the Mean Fiber Population for Each Treatment Group.

which required a special Chi Square procedure (Snedecor, 1956) for the purpose of identifying significant differences in the glycogen depletion pattern among the fiber types within each treatment group. The combined Chi Square value and the source of the significance is illustrated in Tables 5 and 6 for EDL and SOL respectively.

Table 5

Chi Square Analysis for Independence of Fiber Type in Muscle EDL

Treatment Group (%)	Muscle	Fiber Type	% PAS				X^2	Source of Significance
			N	L	M	D		
10	Exp.	FOG	0	24.7	49.8	25.5	14.37*	$D_{FG} > D_{FOG}$
		FG	0	22.0	41.6	36.4		
	Cont.	FOG	0	10.0	55.2	34.8	3.83	
		FG	0	12.0	58.7	29.3		
30	Exp.	FOG	0	18.3	68.0	13.7	27.38*	$D_{FG} > D_{FOG}$
		FG	0.57	22.6	54.7	22.1		
	Cont.	FOG	1.3	9.2	65.6	23.9	6.25	
		FG	1.6	8.2	62.4	27.8		
50	Exp.	FOG	14.6	52.9	30.3	2.2	15.86*	$D_{FG} > D_{FOG}$
		FG	18.0	47.6	29.3	5.1		
	Cont.	FOG	0	9.8	67.0	23.2	5.99	
		FG	0	13.1	60.5	26.4		
70	Exp.	FOG	26.1	56.9	16.5	0.5	13.59*	$N_{FG} > N_{FOG}$
		FG	34.8	46.9	17.6	0.7		
	Cont.	FOG	2.4	10.2	59.2	28.2	7.79	
		FG	0	12.3	56.3	31.4		
100	Exp.	FOG	50.2	42.9	6.9	0	9.49*	$M_{FOG} > M_{FG}$
		FG	54.5	43.6	1.9	0		
	Cont.	FOG	1.2	10.5	54.4	33.9	19.66*	$L_{FG} > L_{FOG}$
		FG	3.2	15.1	56.5	25.2		

* Sig. at $\alpha = .05$.

Table 6

Chi Square Analysis for Independence of Fiber Type in Muscle SOL

Treatment Group (%)	Muscle	Fiber Type	% PAS N	% PAS L	% PAS M	% PAS D	X^2	Source of Significance
10	Exp.	SO	0	17.5	71.1	11.4	8.62*	$D_{FOG} > D_{SO}$
		FOG	0	12.8	71.1	16.1		
	Cont.	SO	0	5.4	70.2	24.4	1.46	
		FOG	0	5.3	67.6	27.1		
30	Exp.	SO	2.9	45.9	49.9	1.3	11.99*	$D_{FOG} > D_{SO}$
		FOG	2.7	40.4	52.5	4.4		
	Cont.	SO	0	7.5	71.5	21.0	31.55*	$L_{FOG} > L_{SO}$ $M_{SO} > M_{FOG}$ $D_{FOG} > D_{SO}$
		FOG	0	12.4	54.8	32.8		
50	Exp.	SO	22.0	53.2	24.2	0.6	9.84*	$N_{FOG} > N_{SO}$
		FOG	27.9	47.3	23.1	1.7		
	Cont.	SO	0.3	18.3	65.2	16.2	6.69	
		FOG	1.1	22.1	59.6	17.2		
70	Exp.	SO	21.9	69.4	8.7	0	2.36	
		FOG	22.4	66.6	11.0	0		
	Cont.	SO	0	9.7	68.7	21.6	4.71	
		FOG	0	12.2	63.1	24.7		
100	Exp.	SO	69.6	30.4	0	0	2.23	
		FOG	65.8	34.2	0	0		
	Cont.	SO	5.4	33.6	51.0	10.0	7.99*	$D_{FOG} > D_{SO}$
		FOG	4.7	28.5	51.3	15.5		

* sig. at $\alpha = .05$.

CHAPTER V

DISCUSSION

Introduction

This section will be presented under the following headings: reliability; number of fibers rated; homogeneity of fiber types; glycogen depletion and stimulation intensity; the significance of glycogen depletion between fiber types as a function of stimulation intensity; and the significance of stimulation-induced glycogen depletion in fast and slow muscle.

Reliability

The Chi Square test for reliability established that the rating procedure was reliable since no significant bias existed in the rating of the serial photomicrographs.

Number of Fibers Rated

Although there were different number of fibers rated per group in each muscle, the proportions of muscle fiber types was not significantly different in EDL and SOL.

The unequal number of fibers rated in each experimental group may be attributed to different muscle sizes, the field chosen to best represent PAS staining intensity of the entire muscle and/or incomplete section of muscle due to freezing artifact and/or tearing in the microtome sections.

Homogeneity of Fiber Types

The proportions of fiber types, expressed as frequency counts, was found to be non-significant across all groups in both muscles as a result of Chi Square analysis. Therefore, the fiber type composition within each muscle group was considered to be homogeneous, despite the different number of fibers rated across the treatment groups.

Glycogen Depletion and Stimulation Intensity

The application of selected voltages to fast and slow mouse muscle resulted in different glycogen depletion patterns as illustrated in Figures 2 and 3 respectively. The effect of increasing stimulation intensity produced an overall reduction in the PAS staining intensity in EDL and SOL. Qualitatively, this would indicate that, as stimulation intensity is increased, more muscle fibers are activated and the degradation of muscle glycogen is increased as noted by the increasing proportion of PAS light and negative-rated fibers in the higher intensity stimulation groups.

The contralateral muscle, which was sham-operated, was used as the control. The PAS staining intensity was fairly uniform across both muscle groups. Approximate PAS ratings were 25% dark, 60% moderate and 15% light. An insignificant percentage of fibers in the Control group were rated PAS negative. The small number of negative fibers were found in the contralateral muscles of mice subjected to the 70 and 100% voltages. This observation would tend to indicate that there was some "cross-over" stimulation effect

present at these higher intensities. Since the afferent nerve supply was not cut in the experimental hindlimb, the high voltages that were directly applied to the muscle may have triggered the afferent nerves thus causing a reciprocal reflex contraction in the contralateral hindlimb. This phenomena was evident more in the FG fibers in EDL and the SO fibers in SOL, however, its overall effect was very minimal comparing the control muscles of the 100% group to that of the 10% group.

The glycogen depletion pattern observed in this study was not identical to the depletion pattern found by other investigators (Edgerton et al., 1970; and Kugelburg and Edstrom, 1968) using direct and indirect stimulation techniques. Experimental protocol differences were most likely to be the causative factor. Both investigators, previously mentioned, utilized supra-maximal voltages in their experiments. As well, their mode of stimulation was via the nerve whereas in the present study, the muscle was directly stimulated. The results of both studies demonstrated that the FG fiber was selectively depleted of its glycogen as a result of the stimulation which would indicate a selective recruitment of the FG fiber population during supra-maximal electrical stimulation. The results of this study, when compared to the findings of the studies of Edgerton et al., (1970) and Kugelburg and Edstrom (1968) must be made with caution as their experimental muscles contained all three fiber types whereas, in the present

investigation, two muscles were used because of the absence of the FG fiber in SOL and the SO fiber in EDL.

Significance of Glycogen Depletion Between Fiber Types

EDL: The Chi Square analysis for independence of fiber type is presented in Table 5. The source of significance is also indicated. In the lower intensity stimulation groups (10, 30, 50%), it would appear, at first glance, that significantly more FG fibers are PAS dark-rated than FOG fibers. However, when analyzing the negative and light PAS categories, the complement does not occur, ie., a higher percentage of FOG fibers would be expected to be glycogen depleted. Consequently, it appears that one particular fiber type does not demonstrate a glycogen depletion pattern that would indicate a selective recruitment at the lower intensities of stimulation.

The glycogen depletion pattern in the higher intensity stimulation groups (70 and 100%) demonstrates a trend towards more FG light-staining fibers. The contralateral control muscles produced a slight PAS lightening effect at 100% compared to the 10% control muscles. The effect was seen more noticeably in the FG fiber population.

SOL: The Chi Square analysis for independence of fiber type is outlined in Table 6. The source of significance is also presented. In the 10 and 30% groups, there were significantly more PAS dark-rated FOG fibers than SO fibers. As well, there existed a greater percentage of

light and negative staining fibers in the SO population although the difference was not significant. This pattern demonstrates a definite lightening trend in the SO fibers at the lower intensities.

At the 50% intensity, a shift in the glycogen depletion pattern was observed. Significantly more FOG fibers were rated PAS negative than SO fibers. This trend, however, was not evident at the higher intensities in SOL. The depletion pattern at 70 and 100% demonstrated that both the SO and FOG fibers were affected approximately in the same proportion.

As occured in EDL, the high voltages of the 100% group caused a mild "cross-over" stimulation effect in the contralateral muscles in SOL. This slight lightening effect in the PAS stain was most noticeable in the SO fibers.

The PAS staining intensity of the 30% control muscles demonstrated significant differences between fiber types. This significance may be attributed to a small number of fibers rated in this group. The number of fibers rated from animals 2 and 4 were very dissimilar which may account for the observed significance. Animals 1 and 3 showed a similar pattern.

Significance of Stimulation-Induced Glycogen Depletion in Fast and Slow Muscle

The depletion of muscle glycogen via direct electrical stimulation in fast and slow muscle may provide valuable insight into the relative frequency with which the three

fiber types contract during exercise. Recent studies by Baldwin et al., 1972; Burke et al., 1971; and Kugelburg and Edstrom, 1968; confirmed that the FG fiber type is selectively depleted of its glycogen stores when subjected to prolonged, intermittent electrical stimulation utilizing supra-maximal voltages. The results of this study indicate that by varying stimulation voltage, fast and slow skeletal muscle of the mouse produced a marked glycogen depletion pattern.

In SOL, at intensities of 10 and 30%, glycogen depletion was more evident in the SO than the FOG fiber population. In EDL, however, the glycogen depletion pattern was inconclusive as it appeared the FOG fibers did not respond in a complementary fashion in the light PAS category to the staining intensity in the dark PAS category. Several factors may account for this observed trend.

Firstly, the low voltages used at these intensities would not cause a significant build up of intramuscular tension which would tend to restrict blood flow through the muscle and hence deprive the fibers of oxygen for energy production. Kjellmer (1964) demonstrated that maximal vasodilation in muscle is reached at a frequency of 5-10/sec. The possibility exists, however, that since the frequencies used in this study exceeded 10/sec in order to produce an unfused tetanus, ischemia may have occurred at the cellular level.

Secondly, during low level contractile activity, very little lactic acid is produced. Although lactic acid

concentration was not investigated here, previous studies involving rodents (Armstrong et al., 1974; Baldwin, 1973) has shown that lactate accumulates exponentially as a function of work intensity. Since the decline in tetanic tension was not marked at these lower intensities, it can be assumed that lactic acidosis was not occurring to any significant degree in the fibers of EDL and SOL.

The conditions created within the muscle as a result of low intensity stimulation, ie., possible ischemia and minimal concentrations of lactate, would favour the function of a fiber population metabolically designed for oxidative phosphorylation and mitochondrial respiration (Baldwin et al., 1977). The significant depletion of glycogen at 10 and 30% in the SO fibers of SOL would support the above statement.

Aside from the metabolic factors, the electrophysiological properties of the different fiber types in skeletal muscle may account for this selective depletion effect in SOL. Campion (1974) investigated the resting membrane potential (RMP) and intra-cellular potassium and sodium concentration of guinea pig hindlimb muscles. He concluded that fast twitch fibers have a higher RMP than slow twitch fibers which may account for the SO fibers being more depleted because those fibers were the first to be depolarized by the electric current.

At the 50% intensity, the trend was towards a lightening in the FG fibers in EDL. The reduction in PAS staining intensity was more marked at the 70 and 100%

intensities. It appears that, as a result of the more intense contractile stimulation, the oxidative fiber pool (FOG) was unable to produce the strength of contraction, hence the muscle's other fiber pool, FG, supplied the contractile force at the higher loads. The possible mechanisms for this "transition" in the glycogen depletion pattern from one fiber type to another will be discussed.

The larger increments in isometric tension seen at intensities of 50% and above are a direct result of more muscle fibers being activated. As a result, the tension intramuscularly may cause vasoconstriction causing a reduced blood flow and diminished oxygen supply to the muscle fibers. Since the oxidative fiber pool is not favoured under hypoxic conditions, the use of another fiber pool which can function anaerobically would be beneficial. It is apparent from the results of this study that in fast mouse muscle, between the intensities of 30 and 70% of maximal tetanic tension, the response shifts towards a more anaerobically specialized fiber type as supported by the enhanced glycogen depletion in the FG fibers in EDL.

The increased contractile activity would increase the lactate output by the contracting fibers (Armstrong et al., 1974; Diamant, 1968; Baldwin, 1973; and Karlson, 1970, 1971). In a study involving rodents, Baldwin (1977) established a high correlation ($r = 0.99$) between the depletion of glycogen in the FG fiber and blood lactate. Furthermore, he noted that the FG fiber maintained a lactate concentration during

exercise that exceeded the blood concentration. It is possible that at some point between 30 and 70%, the same mechanism is occurring in the muscles of this study.

Campion (1974) determined that superficial muscle fibers had lower RMP values than the deeper muscle fibers. One may assume that the higher voltages used in the 70 and 100% groups were sufficient to activate the deeper fibers within the muscle as well as the superficial fibers. The glycogen depletion pattern at 100% supports the view that all fibers were activated.

The observation that more FG fibers are lighter-stained than FOG in EDL at the higher intensities is consistent with the metabolic properties of the FG fiber. The FG fiber has a greater capacity to store glycogen (Gillespie et al., 1970; Beatty, et al., 1963; Dubowitz and Pearse, 1960) and for glycogenolysis (Baldwin et al., 1973; and Peter et al., 1972). Hence the increased rate of glycogen breakdown within the FG fiber would account for the significantly more light and negative PAS staining fibers in FG than FOG. Due to the FOG fibers moderately high glycogenolytic capacity (Baldwin et al., 1973; Peter et al., 1972) and a high capacity to oxidize pyruvate, it would be the preferred fiber type to be used during prolonged stimulation when the glycogen stores in the FG fibers are depleted and the fibers are unable to produce significant amounts of tension.

The depletion of muscle glycogen, which serves as an

important energy source for muscular contraction, has been used by many investigators as an indicator of fiber recruitment in exercise and electrical stimulation studies (Edgerton et al., 1970; Burke et al., 1971; Edgerton and Hewitt, 1972 b; Edgerton and Lehto, 1972 c). Assuming that glycogen depleted fibers were those fibers that had been actively contracting, it is possible to make the following conclusions based on the glycogen depletion pattern found in fast and slow mouse muscle of this study.

Low intensities of electrical stimulation representing 10 and 30% of maximal tetanic tension resulted in the recruitment of significantly more SO fibers than FOG in SOL. In EDL, however, the glycogen depletion pattern was inconclusive and hence no specific recruitment was observed in fast muscle.

Secondly, in EDL, at stimulation intensities somewhere between 30 and 70% of maximal tension, significantly more FG fibers were recruited compared to FOG. This "transition" to the fast glycolytic fiber pool was most marked at 70 and 100%. The response of the SO and FOG fibers to these higher intensities of stimulation in SOL was almost identical which is predictable due to the absence of the FG fiber in SOL.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The purpose of this study was to investigate the effects of selected electrical stimulation intensities representing 10, 30, 50, 70 and 100% of maximal tetanic tension on the glycogen depletion pattern in fast and slow mouse skeletal muscle.

The effect of increasing voltage intensity applied directly to the muscle of EDL and SOL was an overall reduction in the PAS staining intensity in all fiber types. Electrical stimulation intensities in the 10 and 30% groups produced significantly more glycogen depleted fibers in the SO fiber type than FOG in SOL. In EDL, however, the depletion pattern at these lower voltages was inconclusive.

In fast mouse muscle (EDL), at stimulation intensities between 30 and 70% of maximal tetanic tension, a significant trend towards progressively more glycogen depleted FG fibers was observed compared to FOG fibers. This "transition" marked a predominant shift to the glycolytic fiber pool recruitment at higher stimulation intensities in EDL. In SOL, however, neither fiber type (SO or FOG) demonstrated a specific recruitment pattern. This is most likely due to the absence of the FG fiber in this muscle.

Recommendations

Further studies using direct and indirect stimulation techniques in fast and slow skeletal muscle would be recommended. Further investigation to determine more exactly at what point this "transition" to fast fiber recruitment occurs in fast muscle would be useful. A more extensive study into the electrophysiological differences in the membranes of fast twitch and slow twitch muscle fibers may provide added information in the area of muscle fiber recruitment. Finally, a stimulation protocol using a muscle with all three fiber types present would be recommended.

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APPENDICES

APPENDIX A

PROCEDURE FOR CALIBRATION OF GRASS MODEL 7 POLYGRAPH

Procedure for Calibration of Grass Model 7 Polygraph

1. Switch recorder "ON" and set the amplifier channel to "Standby".
2. Allow 20 minutes before switching amplifier to "ON".
3. Set Driver Amplifier to "Calibrate" and adjust baseline with "Baseline" control. Press CAL button and check that deviation is 2cm. If not, adjust "Sensitivity" (and baseline if necessary) so that 2cm. is achieved.
4. Set Driver Amplifier to "Use". Adjust to baseline using "Balance" on Preamplifier. (with stepwise "Sensitivity" control at 1mv/cm.). Press "CAL-2MV" button on Preamplifier and check that the deviation is 2cm. If not, adjust with left "Sensitivity" control (adjust baseline with "Baseline" if necessary). Once calibrated, recheck that the baseline does not change when the "Polarity" switch on the Driver Amplifier is switched back and forth from CAL to USE. If it does move, adjust with "Balance".
5. Set to "USE" and proceed with experiment.

APPENDIX B

PROCEDURE FOR MOUSE RINGERS SOLUTION

Mouse Ringers Solution

NaCl	121.0mM
KCl	4.75mM
$NaHCO_3$	25.0mM
KH_2PO_4	0.5mM
$CaCl_2$	1.5mM
$MgCl_2$	9.23mM
glucose	2.0g/l

Bubble with 95% oxygen and 5% carbon dioxide.

APPENDIX C

CHI SQUARE ANALYSIS OF TOTAL FIBERS IN EDL AND SOL

Table 7

Chi Square Analysis for Total Fibers

EDL

Group		o	e	(o-e)	$(o-e)^2$	$\frac{(o-e)^2}{e}$	X^2
10X	FOG	215	261	46	2116	8.1	16.0*
	FG	245	293	48	2304	7.9	
10C		221	261	40	1600	6.1	21.9*
		225	293	68	4624	15.8	
30X		278	261	17	289	1.1	11.8*
		349	293	56	3136	10.7	
30C		314	261	53	2809	10.8	36.6*
		380	293	87	7569	25.8	
50X		314	261	87	7569	29.0	35.9*
		338	293	45	2025	6.9	
50C		264	261	3	9	0.03	.04
		291	293	2	4	0.01	
70X		243	261	18	324	1.2	2.6
		273	293	20	400	1.4	
70C		255	261	6	36	0.14	2.47
		268	293	25	625	2.33	
100X		237	261	24	576	2.2	6.6*
		257	293	36	1296	4.4	
100C		248	261	13	169	0.65	1.42
		278	293	15	225	0.77	

* sig at α = .05

Table 8

Chi Square Analysis for Total Fibers

SOL

Group		o	e	(o-e)	$(o-e)^2$	$\frac{(o-e)^2}{e}$	X^2
10X	SO	330	336	6	36	0.11	1.00
	FOG	379	361	18	324	0.89	
10C		349	336	13	169	0.50	0.52
		358	361	3	9	0.02	
30X		381	336	45	2025	6.03	11.64*
		406	361	45	2025	5.61	
30C		267	336	69	4761	14.16	24.81*
		299	361	62	3844	10.65	
50X		323	336	13	169	0.50	0.59
		355	361	6	36	0.09	
50C		322	336	14	196	0.58	0.98
		349	361	12	144	0.40	
70X		425	336	89	7921	23.57	32.89*
		419	361	58	3364	9.32	
70C		329	336	7	49	0.15	0.95
		344	361	17	289	0.80	
100X		372	336	36	1296	3.86	9.72*
		407	361	46	2116	5.86	
100C		259	336	77	5929	17.64	28.63*
		298	361	63	3969	10.99	

* sig at $\alpha = .05$

APPENDIX D

CHI SQUARE TEST FOR HOMOGENEITY OF FIBERS IN EDL AND SOL (PROPORTIONS EXPRESSED AS FREQUENCY COUNTS)

Table 9

Chi Square Test for Homogeneity of Fibers
(Proportion of fibers expressed as frequency counts)

EDL FOG - 47%, FG - 53%

Group		o	e	(o-e)	$(o-e)^2$	$\frac{(o-e)^2}{e}$	X^2
10X	FOG	215	216	1	1	.004	.008
	FG	245	244	1	1	.004	
10C		221	210	11	121	.58	1.09
		225	236	11	121	.51	
30X		278	295	17	289	.98	1.85
		349	332	17	289	.87	
30C		314	326	12	144	.44	.83
		380	368	12	144	.39	
50X		314	306	8	64	.21	.39
		338	346	8	64	.18	
50C		264	261	3	9	.03	.06
		291	294	3	9	.03	
70X		243	243	0	0	0	0
		273	273	0	0	0	
70C		274	269	5	25	.09	.17
		298	303	5	25	.08	
100X		237	232	5	25	.11	.20
		257	262	5	25	.09	
100C		248	247	1	1	.004	.007
		278	279	1	1	.003	

* sig at $\alpha = .05$

Table 10

Chi Square Test for Homogeneity of Fibers
(Proportion of fibers expressed as frequency counts)

SOL SO - 48%, FOG - 52%

Group		o	e	(o-e)	$(o-e)^2$	$\frac{(o-e)^2}{e}$	X^2
10X	SO	330	340	10	100	.29	.56
	FOG	379	369	10	100	.27	
10C		349	339	10	100	.29	.56
		358	368	10	100	.27	
30X		381	378	3	9	.02	.04
		406	409	3	9	.02	
30C		267	272	5	25	.09	.18
		299	294	5	25	.09	
50X*		323	325	2	4	.01	.02
		355	353	2	4	.01	
50C		322	322	0	0	0	0
		349	349	0	0	0	
70X		425	405	20	400	.98	1.89
		419	439	20	400	.91	
70C		329	323	6	36	.11	.21
		344	350	6	36	.10	
100X		372	374	2	4	.01	.02
		407	405	2	4	.01	
100C		259	267	8	64	.24	.46
		298	290	8	64	.22	

* sig at $\alpha = .05$

APPENDIX E

FREQUENCY DATA OF PAS STAINING INTENSITY OF MUSCLE FIBERS

Table 11

PAS Staining Intensity of
Mouse EDL Muscle Fibers (Frequency Counts)

	STIMULATED MUSCLE PAS STAINING INTENSITY								CONTROL MUSCLE							
	FOG				FG				FOG				FG			
	N	L	M	D	N	L	M	D	N	L	M	D	N	L	M	D
10_1	0	7	10	18	0	5	16	23	0	7	25	13	0	6	32	5
10_2	0	6	29	17	0	4	31	24	0	3	35	29	0	8	48	23
10_3	0	28	31	9	0	36	27	7	0	6	21	29	0	11	23	16
10_4	0	12	37	11	0	9	28	35	0	6	41	6	0	2	29	22
30_1	0	16	42	11	0	28	57	8	0	15	52	15	0	18	73	21
30_2	0	7	35	5	0	25	54	12	0	4	63	14	0	0	61	32
30_3	0	10	55	8	0	3	51	23	0	4	38	30	0	2	59	26
30_4	0	18	57	14	2	23	29	34	4	6	53	16	6	11	44	27
50_1	12	47	29	0	10	35	31	8	0	13	73	4	0	9	66	10
50_2	9	36	20	3	12	41	28	2	0	5	27	38	0	8	42	29
50_3	11	56	13	4	20	49	18	7	0	4	19	7	0	6	24	17
50_4	14	27	33	0	19	36	22	0	0	4	58	12	0	15	44	21
70_1	17	31	5	0	34	19	1	0	0	6	15	21	0	7	33	16
70_2	19	52	17	0	27	36	13	2	4	7	51	9	0	3	46	21
70_3	23	35	7	1	19	26	17	0	0	8	53	5	0	14	47	5
70_4	9	30	14	0	15	47	17	0	2	5	32	37	0	9	25	42
100_1	28	35	3	0	32	40	4	0	0	9	21	26	0	3	36	14
100_2	26	43	5	0	39	33	0	0	0	3	23	16	0	4	38	14
100_3	24	9	7	0	37	14	1	0	0	2	44	9	0	19	31	13
100_4	39	13	1	0	32	25	0	0	3	12	47	33	9	16	52	29

Table 12

PAS Staining Intensity of
Mouse SOLEUS Muscle Fibers (Frequency Counts)

	STIMULATED MUSCLE								CONTROL MUSCLE							
	SO				FOG				SO				FOG			
	N	L	M	D	N	L	M	D	N	L	M	D	N	L	M	D
10_1	0	6	75	13	0	10	79	22	0	0	74	26	0	3	67	39
10_2	0	27	52	11	0	17	77	16	0	8	67	10	0	5	61	14
10_3	0	10	43	0	0	14	56	3	0	0	46	22	0	3	51	30
10_4	0	8	37	9	0	3	32	14	0	11	58	27	0	8	63	14
30_1	2	40	43	3	0	37	61	7	0	4	56	18	0	7	41	26
30_2	0	62	35	0	0	70	29	4	0	12	40	16	0	19	32	27
30_3	9	51	26	0	11	43	33	0	0	2	28	9	0	6	31	23
30_4	0	22	86	2	0	14	90	7	0	2	67	13	0	5	60	22
50_1	21	33	6	0	32	27	11	0	0	9	43	24	4	20	39	17
50_2	21	46	17	2	24	52	13	0	0	18	45	14	0	20	49	17
50_3	18	54	27	0	25	46	32	5	0	12	56	2	0	23	49	0
50_4	11	39	30	0	18	43	26	1	1	20	67	12	0	14	71	26
70_1	46	74	8	0	51	66	13	0	0	7	81	13	0	11	69	15
70_2	14	73	6	0	19	64	3	0	0	16	84	7	0	12	77	15
70_3	19	63	7	0	13	71	11	0	0	9	25	29	0	19	27	24
70_4	14	85	16	0	11	78	19	0	0	0	36	22	0	0	44	31
100_1	67	22	0	0	71	31	0	0	0	28	16	3	0	19	23	12
100_2	51	26	0	0	66	20	0	0	0	16	64	8	0	22	58	5
100_3	73	23	0	0	67	31	0	0	7	27	10	0	11	23	24	0
100_4	68	42	0	0	64	57	0	0	7	16	42	15	3	21	48	29

APPENDIX F

HISTOCHEMICAL TECHNIQUE FOR MYOSIN ATPase

MYOSIN ADENOSINE TRIPHOSPHATASE STAIN (ATPase)

- Procedure of Padykula and Herman, as modified by Guth and Samaha. Exp. Neurol. V 25, p. 138-152, 1969.

SOLUTIONS: * volumes are indicated, concentrations are final

I Fixative - * made up to 20 ml.

a) 5 % Formaldehyde (analar chemicals)
1.0 ml. into 20 ml.

b) Sucrose (m.w. 342.3)
2.3275 gr. into 20 ml. (340.mM)

c) Calcium Chloride (m.w. 110.99)
0.1509 gr. into 20 ml. (68.0 mM)

d) Sodium Cacodylate (m.w. 214.02)
0.856 gr. into 20 ml. (200.0 mM)

** make up to 20 ml. with distilled water.
** pH taken to 7.6 with HCl. (in cold - 4°C).
** To be made up fresh.

II Fixative Rinse: Cold (4°C) Distilled Water.

III Pre-incubation Medium: * made up to 25 ml.

a) Calcium Chloride
0.1 gr. into 25 ml. (36.0 mM)

b) 2, amino-2, methyl-1, propanol (m.w. 89.14)
12.5 ml. of 0.2 M stock buffer into 25 ml. (0.1 M)

** make up to 25 ml. with distilled water.
** pH adjusted to 10.4 with HCl (in cold).
** To be made up fresh.

IV Incubation Medium: * made up to 25 ml.

a) Propanol Buffer
12.5 ml. of 0.2 M stock buffer into 25 ml. (0.1 M)

b) Calcium Chloride
0.0499 gr. into 25 ml. (18.0 mM)

c) Disodium ATP (m.w. 551.2)
0.062 gr. into 25 ml. (4.5 mM)

** Make up to 25 ml. with distilled water.
** pH adjusted to 9.4 with HCl.
** To be made up fresh.

V 0.2 M Buffer Stock: * make up to 75 ml.

a) 1.3371 gr. of 2, amino-2, methyl-1, propanol buffer into 75 ml. (0.2 M)

VI 0.1 M. Buffer Rinse: * make up to 100. ml.

a) 50 ml. of 0.2 M stock diluted with 50. ml. of distilled water (0.1 M)

** pH adjusted to 9.4 with HCl
** To be made up fresh.

VII 1% Calcium Chloride:
1.0 gr. $CaCl_2$ in 100. ml. of distilled water

VIII 2% Cobaltous Chloride: (m.w. 237.95)
2.0 gr. $CoCl_2$ in 100. ml of distilled water

IX 1% Ammonium Sulphide: (assay 23.4%)
1.0 ml. $(NH_4)_2S$ in 99. ml. of distilled water.

NB: Above solutions VII, VIII, and IX must be stored in brown glass bottles at 4°C if made up as stock.

MYOSIN ATPase PROCEDURES

1) Fixative - 10 min in cold ($4^{o}C$) fixative medium
2) Wash - 4 x 3 min cold distilled water rinse
3) Pre-incubation - 15 min. in cold pre-inc. medium
4) Incubation - 45 min in $37^{o}C$ incubation medium
5) 1% $CaCl_2$ - 3 x 30 sec rinse
6) Drain and blot
7) 2% $CoCl_2$ - 1 x 3 min wash
8) 0.1 M buffer rinse - 4 x 30 sec rinse (re-establish pH)
9) 1% $(NH_4)_2S$ (Ammonium sulphide) - 1 x 3 min
10) Tap water rinse (cold) - 1 x 5 min
11) Ascending alcohols - ethanol
    95% alcohol - 1 x 1 min
    99% alcohol - 2 x 1 min
12) Xylene - 1 x 2 min
13) Mount - Permount

APPENDIX G

ELECTRICAL STIMULATION DATA OF MUSCLES EDL AND SOL

Table 13

Electrical Stimulation Data - Muscle EDL

Animal No.	Body Weight (gr)	Max Voltage (Volts)	Max Twitch Tension (gr)	Max Tetanic Tension (gr)	Exp. Voltage
10 - 1	23.0	60	5.0	12.0	2.0
- 2	20.5	40	6.0	24.5	1.6
- 3	26.0	40	7.5	27.0	1.3
- 4	23.0	60	4.5	16.0	3.9
30 - 1	17.0	60	6.5	14.5	1.8
- 2	19.0	70	7.0	20.0	3.1
- 3	19.5	40	4.0	10.0	3.0
- 4	23.5	40	6.0	16.0	3.8
50 - 1	21.5	50	6.5	22.0	14.0
- 2	22.5	50	7.0	23.0	8.0
- 3	20.5	30	7.5	20.0	9.0
- 4	25.0	70	6.5	27.0	9.5
70 - 1	26.0	50	4.5	12.0	32.0
- 2	20.5	50	7.0	21.0	28.0
- 3	23.5	50	6.0	15.0	40.0
- 4	24.5	60	5.0	22.0	41.0
100 - 1	27.0	50	5.75	23.0	50
- 2	24.5	60	6.0	14.5	60
- 3	29.0	60	5.5	16.0	60
- 4	28.0	30	7.0	24.0	40

Table 14

Electrical Stimulation Data - Muscle SOL

Animal No.	Body Weight (gr)	Max Voltage (Volts)	Max Twitch Tension (g)	Max Tetanic Tension (g)	Exp. Voltage
10 - 1	24.5	50	2.7	9.0	2.2
- 2	23.0	50	3.0	10.0	1.8
- 3	23.5	70	3.3	8.0	2.0
- 4	21.0	70	3.4	10.0	1.3
30 - 1	25.0	60	3.1	11.0	4.6
- 2	23.5	50	2.3	7.3	3.5
- 3	24.0	60	1.7	4.8	3.0
- 4	27.0	70	2.4	8.5	3.9
50 - 1	23.0	70	3.2	9.0	16.0
- 2	25.5	50	2.8	9.5	24.0
- 3	26.0	60	2.0	6.5	20.0
- 4	29.0	60	3.0	9.8	28.0
70 - 1	27.5	60	2.5	9.3	50.0
- 2	24.5	60	3.3	10.3	55.0
- 3	27.0	70	2.6	9.5	54.0
- 4	23.0	50	2.8	10.0	42.0
100 - 1	26.0	50	2.4	8.4	50.0
- 2	28.5	60	3.1	9.2	60.0
- 3	30.5	60	2.5	10.0	60.0
- 4	27.5	60	2.2	7.9	60.0