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IN MEMORY OF MY FATHER

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LIST OF DEFINITIONS

ATP = adenosinetriphosphate

ADP = adenosinediphosphate

Cr = creatine

CK = creatine kinase

CrP = creatine phosphate

$-dG/d\xi$ = free energy change of ATP hydrolysis

FDNB = fluorodinitrobenzene

GP = β-guanidinopropionic acid

GPP = phosphorylated β-guanidinopropionic acid

MLCP = myosin light chain phosphorylation

PTD = posttetanic twitch depression

PTP = posttetanic twitch potentiation

T = peak isometric tension

$+\dot{T}$ = maximum rate of tension development

$-\dot{T}$ = maximum rate of relaxation

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ABSTRACT

The creatine analogue β-guanidinopropionic acid was administered to weanling rats as 1% of their diet for approximately 30 days to establish the possible role of the creatine-creatine phosphate system in skeletal muscle contraction. Total muscle creatine and creatine phosphate levels were reduced by 80% and 96%, respectively, and ATP content decreased by 64-76% as measured from extracts of frozen EDL muscles.

Peak isometric twitch tension and the derivative of tension remain unchanged by creatine depletion. The only change in the single brief tetanus is a slowing in the rate of relaxation.

After 1 second of tetanic stimulation the maximum rate of tension development (+$\dot{T}$) of a subsequent tetanus increases by nearly 20% in normal muscle *versus* a 30% reduction in depleted muscle. The potentiation lasts minutes while the depression disappears in seconds.

Twitch tension and the derivative of tension following 1 second of tetanic stimulation is almost doubled in normal muscles. Again the potentiation lasts minutes. Depleted muscles exhibit a biphasic response to this pattern of stimulation. An early depression of the twitch occurs which

recovers within 1 second and is followed by a small potentiation.

Increasing the degree of activation by delivering multiple pulses after the 1 second tetanus reduced both the potentiation in normal muscles and the early depression in depleted muscles.

It appears that the activation and deactivation steps of contraction are altered following a 1 second tetanus in creatine depleted muscles. This is likely due to an effect of reduced levels of energy substrates on calcium dynamics. We are proposing that the creatine-creatine phosphate system functions primarily to buffer changes in the ATP/ADP ratio.

INTRODUCTION

1. Historical Background

Since its discovery in 1927 by both Eggleton and Eggleton and Fiske and Subbarow creatine phosphate, then called phosphagen, has been the subject of extensive research and many ideas have been put forward to explain its role in muscle contraction. Fiske and Subbarow (1929a) observed that creatine phosphate breakdown is accompanied by a marked increase in pH, also shown by Meyerhof and Lohmann (1928), and proposed that this mitigates fatigue by neutralizing lactic acid. Lohmann (1929) and Fiske and Subbarow (1929b) independently discovered an adenylate compound which dramatically altered the picture of metabolism in resting and contracting muscles. This substance, now called adenosinetriphosphate (ATP), is fundamental for energetics of all cells.

In the following year Lundsgaard (1930) found that creatine phosphate breakdown will not occur in the absence of adenylate compounds. As a result of his work the following reaction was elucidated (the Lohmann reaction):

$$\mathrm{CrP} + \mathrm{ADP} \rightleftharpoons \mathrm{ATP} + \mathrm{Cr}$$

which is catalyzed by the enzyme creatine kinase (CK).

During the ensuing years there was controversy over the respective roles of creatine, creatine phosphate, lactic acid, and ATP in muscle contraction. Because no one was able to show a change in ATP levels during activity, creatine phosphate seemed to be the direct energy source for muscle contraction, however Szent-Györgyi (1945) and Engelhardt and Lyubimova (1939) had shown that ATP, and not CrP, acts upon isolated actomyosin. It wasn't until 1962 when Cain and Davies used fluorodinitrobenzene (FDNB) to inhibit the enzyme creatine kinase that ATP breakdown during a single contraction was demonstrated and its role as a primary energy source was unequivocally established.

Once it became certain that creatine phosphate is not the direct energy donor, the hypothesis emerged that it functions as a high energy phosphate reservoir. It was reasoned that ATP is rapidly used during contraction and must be rapidly replaced. As ADP is formed it is rephosphorylated by a transfer of phosphate from creatine phosphate. However, it was not clear just how creatine phosphate was regenerated following this phosphoryl transfer to ADP.

A mitochondrial bound isozyme of creatine kinase which is different from the cytoplasmic enzyme was soon reported in rat skeletal muscle, heart and brain (Jacobs *et al.*, 1964; Swanson, 1967) which led to the first suggestion that creatine phosphate could function as a "carrier" of high energy phosphate from the mitochondria to the energy utilizing sites. The mitochondrial creatine kinase could maintain the intracellular concentration of creatine phosphate because it is located near sites of ATP formation where the local ATP/ADP ratio would favour the production of creatine phosphate. Creatine phosphate would then be utilized at the myofibrils to maintain ATP levels through the reverse reaction, catalyzed by the sarcoplasmic isozyme, which favours creatine phosphate utilization (Swanson, 1967). This theory provides a neat answer to the question of creatine phosphate regeneration. It also requires the existence of a functional subcellular ATP compartmentation, a hypothesis proposed by Gudbjarnason *et al.* (1970) to account for their observation that ischemic dog heart muscle stopped contracting when 70% of the creatine phosphate had been hydrolyzed yet 80% of the ATP was still present.

Further support for the energy shuttle theory was provided by the identification of a myofibrillar creatine kinase isozyme bound to the M-line of chicken skeletal muscle (Turner *et al.*, 1973; Walliman *et al.*, 1977).

Bessman and Fonyo (1966) isolated pigeon breast muscle mitochondria and varied the levels of creatine and adenine nucleotides in the media. They found that creatine can exert a respiratory control in these isolated mitochondria and proposed that creatine could function in a feedback regulation of respiration in response to muscular activity which could be mediated through the shuttle of creatine and creatine phosphate. This suggestion was also proposed by Jacobus and Lehninger (1973) from their work on rat heart mitochondria.

The observation that creatine phosphate synthesis is inhibited by atractyloside (a nucleotide transport inhibitor) in respiring isolated mitochondria (Jacobus and Lehninger, 1973) and the kinetic evidence for the juxtaposition of mitochondrial creatine kinase and ATP-ADP translocase (Saks *et al.*, 1976) led to the suggestion that mitochondrial creatine kinase is coupled to oxidative phosphorylation. Radioactive tracer studies showing that mitochondrial creatine kinase can utilize mitochondrially-generated ATP more effectively than exogenous ATP (Yang *et al.*, 1977) strengthen the hypothesis that a tight functional coupling exists between the two enzymes. For additional information on the development of and the evidence for the creatine phosphate shuttle hypothesis the reader should consult the recent reviews by Saks *et al.* (1978), Jacobus (1980), and Bessman and Geiger (1981).

Thus, abundant biochemical evidence is available to support the view that the creatine kinase system provides a mechanism for the transport of metabolic energy from its site of production to its site of utilization as well as for the maintenance of local pools of ATP. Still to be worked out is the question, how much high energy phosphate generated from mitochondrial oxidative phosphorylation is channelled into creatine phosphate formation before leaving the mitochondria *in vivo*? In other words, how essential is this transport system, if it exists, to muscle cell function?

The physiological data provides conflicting evidence in answer to this question. Several important experiments have been performed in which creatine phosphate levels were correlated with the ability to develop tension in frog sartorius (Seraydarian *et al.*, 1961) and mouse soleus (Spande and Schlottelius, 1970) muscles during fatigue, as well as in ischemic *in situ* dog heart (Gudbjarnason *et al.*, 1970) and hypoxic isolated rat heart (Dhalla *et al.*, 1972). The relaxation rate in frog gastrocnemius muscle was also found to be correlated with creatine phosphate level's following a fatiguing stimulus (Dawson *et al.*, 1980).

In the above fatigue and hypoxia experiments, the muscles all stopped contracting when creatine phosphate levels fell to a low value (*circa* 20%) yet the ATP levels had not even decreased by half. Cain and Davies (1962) report that in FDNB poisoned frog muscle, only three full contractions can be elicited even though nearly half the resting ATP concentration remains after two contractions. These experiments suggest that muscle function is dependent upon creatine phosphate and its oxygen-dependent regeneration at the mitochondria and are in support of the energy shuttle theory as well as the subcellular compartmentation of ATP.

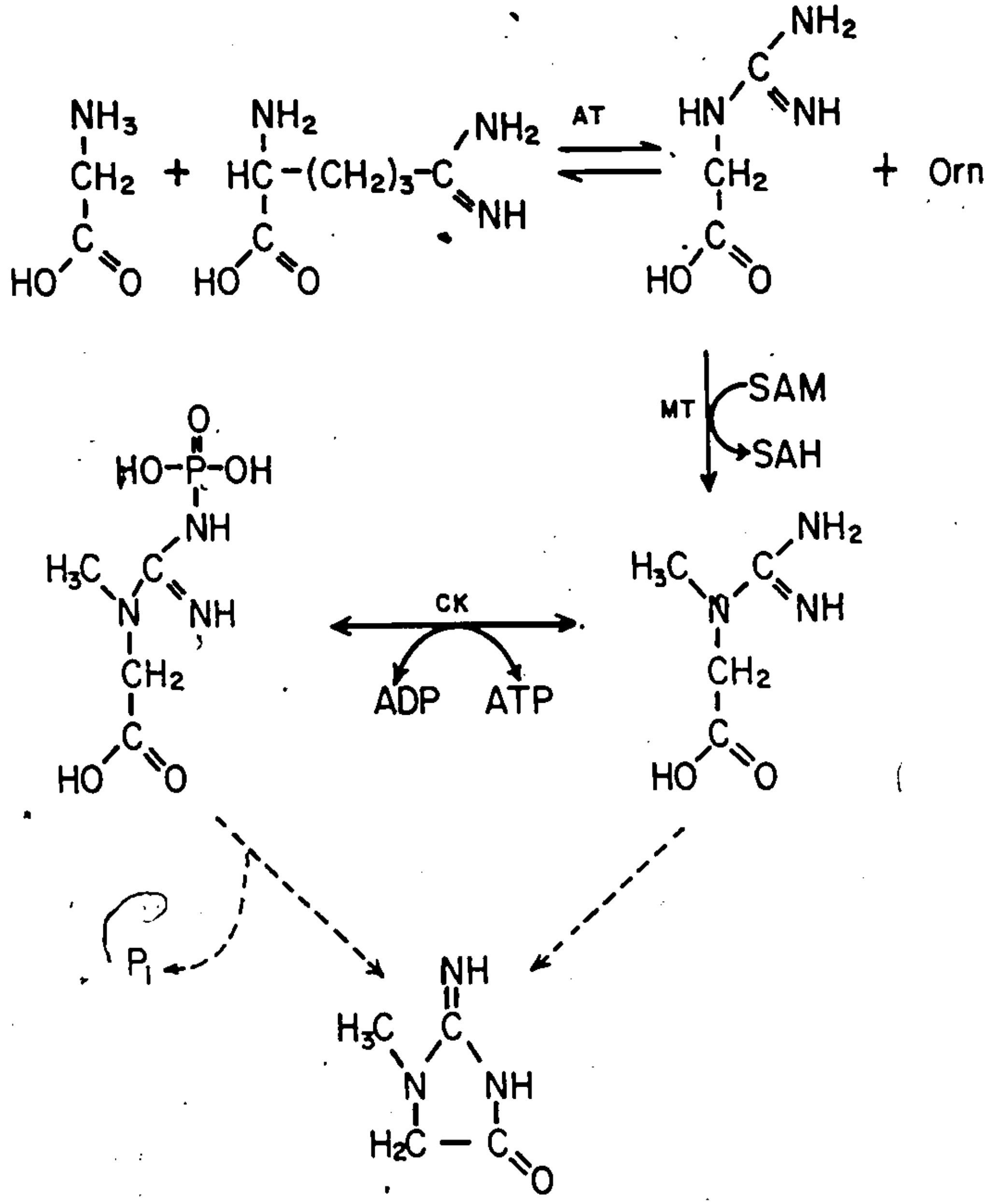
When creatine phosphate levels are decreased by the method of creatine analogue feeding however, the effect on muscle function is not clear cut.

2. Creatine biochemistry and the development of analogue feeding

Before describing the technique of creatine analogue feeding, something should be said about creatine metabolism. The existence of creatine was discovered as early as 1835 in meat extract (Chévreul, 1835) and its importance in muscle contraction was suspected even before the discovery of creatine phosphate or ATP. Much of the early work on creatine was concerned with the sites and the mechanism of its synthesis. In 1941 Bloch and Schoenheimer conducted a comprehensive investigation with labelled putative precursors and found that guanidinoacetate is synthesized *in vivo* from arginine and glycine. As shown in Figure 1., creatine biosynthesis is a two-step pathway. First is a reversible transfer of an amidino group from arginine to glycine to form guanidinoacetate and the second is an irreversible transfer of a methyl group from S-adenosylmethionine (SAM) to guanidinoacetate to form creatine (Bloch and Schoenheimer, 1941; Borsook and Dubnoff, 1941; Cantoni and Vignos, 1954).

The two enzymes involved in creatine biosynthesis have

Figure 1. Creatine biosynthesis. The top line shows the formation of guanidinoacetate and ornithine from glycine and arginine and the methyl transfer from S-adenosylmethionine (SAM) to form creatine is directly below. The bottom part shows the interconversion of creatine and creatine phosphate and and their cyclization to creatinine.


AT
Orn
MT
SAM
SAH
CK
ADP
ATP
P_i

species-specific organ distributions (Walker, 1979) but in general, mammals have high levels of the first enzyme, amidinotransferase (AT), in the kidney and high levels of the second enzyme, methyltransferase (MT), in the liver. The pancreas contains high levels of both enzymes. The enzymes of creatine synthesis are not found in tissues that use creatine (muscle and brain) and the enzyme that phosphorylates creatine, creatine kinase, is not found in tissues that synthesize it. This separation of sites of creatine synthesis and creatine phosphate synthesis and utilization allows independent regulation of each process. Regulation evidently does occur because the output of creatinine (the only known end product of creatine and creatine phosphate metabolism) from the body pool is relatively constant at about 2 grams per day (Walker, 1979).

Once the pathways and sites of creatine biosynthesis became fairly well established, interest was focused on the uptake and retension of creatine by muscle. Fitch and Shields (1966) investigated creatine movement across rat EDL muscles *in vitro* using ^{14}C-creatine and found that the entry process is saturable, indicating a carrier-mediated transport system. A subsequent study by the same authors investigated substrate specificity and inhibition properties of this transport system (Fitch *et al.*, 1968). A variety of compounds were tested for their ability to inhibit creatine entry and of those that had no effect, none possessed an amidino group. Of those that inhibit entry, β-guanidinopropionic acid (GP) is the most effective and is shown to be competitive. The V_{max} of GP is identical to that of creatine and K_m is less; 0.2 mM for GP compared to 0.5 mM for creatine (Fitch *et al.*, 1968).

Creatine and GP have exactly the same molecular weight and their structure differs only by a methyl group that is present as a side branch of the main chain in creatine but within the main chain in GP. The similarity of these two compounds and the competitive nature of the inhibition that was observed suggest that creatine and GP compete for the same substrate-specific transport site for entry into skeletal muscle. The K_m values show GP to be the better competitor which led to the use of this compound in feeding trials as a method of depleting muscle creatine *in vivo* (Shields and Whitehair, 1973). Injection of ^{14}C-creatine into rats fed 1% GP in their diet for 50 days showed reduced entry of creatine into muscle tissue and increased urinary excretion of creatine. Total muscle creatine was reduced by 75% of its normal value and the urinary creatine/creatinine ratio was elevated about 7-fold.

This study was followed up by Fitch and his co-workers (1974) who also reported the total creatine content of rat gastrocnemius muscles to be decreased by 75% after 6 weeks of feeding with 1% GP. The creatine phosphate content was decreased by 93%. Examination of organic phosphates by chromatography revealed the existence of a new phosphorylated compound in extracts of creatine depleted muscle. This new compound was indistinguishable from phosphorylated GP (GPP) formed *in vitro* and was very slowly hydrolyzed by creatine kinase under assay conditions. To examine this further, gastrocnemius muscles were stimulated to contract under anoxic conditions and both creatine phosphate in normal muscle and GPP in depleted muscle decreased dramatically. This suggested that GPP can substitute functionally for creatine phosphate. Other observations worthy of mention are the reduced concentrations of glucose-6-phosphate, ADP, and ATP in resting muscle from rats fed 1% GP, by 54%, 25% and 52%, respectively (Fitch *et al.*, 1974).

No major histological changes were observed in any tissues from rats fed 1% GP (Shields *et al.*, 1975) although their fast twitch muscle fibres appeared smaller (Shields *et al.*, 1975; Fitch *et al.*, 1978; Petrofsky and Fitch, 1980).

Using an *in situ* preparation of tibialis anterior muscle from GP-fed rats, it was again shown that anoxic contracting muscles break down GPP (Fitch *et al.*, 1975). These authors estimated that the total use of high energy phosphate (~P) was reduced by 32% in depleted muscles compared to normal and the reduction of total tension development in these muscles followed closely at 28%. These results suggest that GP can substitute functionally for creatine phosphate but that it may be a relatively poor substrate for creatine kinase. It is worth noting that during the repetitive stimulation, control muscles exhibited the staircase phenomenon while depleted muscles did not. A possible explanation for this result given by the authors is that ATP concentrations at the cross-bridge sites may be limiting in these muscles since the resting ATP levels are reduced in the presence of high GP.

A different result was obtained in the same laboratory several years later. This time using rat soleus and plantaris muscles, Fitch *et al.* (1978) reported that although GPP accumulates to concentrations of 20-25 μmolg^{-1} which is comparable to the measured level of creatine phosphate in normal muscle (Fitch *et al.*, 1974), less than 12% of it is hydrolyzed during a fatiguing stimulus in both soleus and plantaris muscles. Creatine phosphate levels were decreased by 68-70% in normal muscles during this stimulation procedure. Clearly then, GPP does not serve as an important energy reservoir under these conditions. In a subsequent *in vitro* biochemical investigation, Chevli and Fitch (1979) showed that the V_{max} of creatine kinase activity for GPP is less than 0.1% that for creatine phosphate.

A surprising result was that although the maximum isometric tension (expressed as g/g muscle weight) produced by both the soleus and plantaris muscles was not significantly affected by GP feeding, the endurance of the depleted soleus was markedly prolonged. These muscles could maintain 70% of their maximum isometric tension for 106 ± 40 seconds while normal soleus for only 43 ± 18 seconds. Depleted plantaris muscles showed no significant change in endurance (Fitch *et al.*, 1978).

Petrofsky and Fitch (1980) extended these observations in a subsequent experiment using an elegant stimulation technique developed by Petrofsky (1979). In contrast to the previous investigation, depleted soleus muscles developed only 61% of the normal twitch tension and both the rise time and half relaxation time were significantly prolonged. Again, the isometric endurance of creatine depleted soleus muscles was prolonged by a factor of nearly three, while this parameter did not change in depleted plantaris muscles. If the creatine-creatine phosphate shuttle does exist, it appears not to be essential for the maintenance of a steady state work rate.

Perhaps creatine phosphate hydrolysis during aerobic muscle contraction serves some other purpose than to provide energy to sustain contraction. No absolute differences were observed between control and depleted plantaris muscles except that the depleted muscles are smaller. The slow twitch muscles appear to get slower and the fast twitch muscles appear to get smaller as a result of creatine phosphate depletion.

To more critically evaluate the *effective* energy reserves for contraction in normal and creatine depleted muscles, Mainwood *et al.* (1982a) examined contractile performance after inhibiting respiration and glycolysis. The *apparent* energy reserve (CrP + ATP) was estimated to be 79% depleted in rat diaphragm muscles following GP feeding for four weeks. Following application of 2 mM NaCN and 1.7 mM iodoacetic acid, normal muscles continued to respond to tetanic stimulation for about 16-18 contractions (although the tension development was 80% of the preinhibition level) while the creatine depleted muscles gave only 3 or 4 contractions before going into a state of contracture. Based on the contraction results, the effective energy reserves appear to be reduced by about 74%. Thus the decrease in energy reserves seems to correspond to the decrease in CrP and ATP in creatine depleted diaphragm muscles indicating once again that GPP is not a functional energy reserve.

In a subsequent study Mainwood *et al.* (1982b) compared the contractile parameters of normal and depleted diaphragm muscles during a twitch and a brief tetanus and examined the influence of creatine phosphate depletion on the response to both fatiguing stimulation and brief bursts (0.2 second 100 Hertz tetani at 2 per second) of activity. Time to peak tension and half relaxation time in a single twitch were both significantly prolonged as reported by Petrofsky and Fitch (1980) for depleted soleus muscles. The rate of relaxation following a tetanus was also significantly prolonged in depleted diaphragm muscles. Surprisingly, the creatine phosphate depleted muscles exhibited less depression of tension during fatigue and recovered somewhat faster. This corroborates the earlier findings that fatigue does not seem to be dependent on creatine phosphate levels (Fitch *et al.*, 1978, Petrofsky and Fitch, 1980). A very striking effect of creatine phosphate depletion was observed on the velocity of tension development during the brief bursts of activity. The rate of rise of tension development progressively increased in normal muscles over a series of seven contractions while in depleted muscles it sharply declined. The maximum effect seemed to be reached by about 3 seconds of stimulation. ATP levels were found to be unchanged after 3 seconds of stimulation in both normal and depleted muscles indicating that the decline in the rate of tension development in depleted muscles is not a consequence of low muscle ATP. It is still possible that the ATP level is very low in the vicinity of the cross-bridges but, as the authors state, this would imply a restricted ATP mobility and a dependence on creatine phosphate, the latter of which was ruled out by the endurance experiments of Fitch *et al.* (1978) and Petrofsky and Fitch (1980).

In summary then, the earlier biochemical data suggests a physiological role for creatine phosphate in transport of high energy phosphate from source to sink and in buffering local changes in [ADP] and [ATP]. The energy shuttle theory is supported by kinetic studies on mitochondria which suggest a possible coupling between CK and ADP-ATP translocase. Physiological data obtained from fatiguing, hypoxic, and CK-inhibited muscles in general show a dependence of contractile performance on creatine phosphate levels which indirectly supports the above theory. Information obtained from muscles depleted of creatine phosphate by the method of creatine analogue feeding does not indicate a dependence of contraction on creatine phosphate.

These results taken together suggest that creatine phosphate may not perform an essential energy transport function in skeletal muscle. Its importance as an immediate and dynamic energy reserve to buffer changes in [ATP] and [ADP] during brief bursts of activity is suggested by the recent work on diaphragm muscle.

STATEMENT OF PURPOSE

The fast twitch rat extensor digitorum longus (EDL) muscle, which consists of 59% fast oxidative-glycolytic, 38% fast glycolytic, and 3% slow oxidative muscle fibres (Ariano et al., 1973), is known to have a high rate of ATP utilization (Wendt and Gibbs, 1973). If the CrP-Cr system really functions as a dynamic buffer during sudden changes in energy demand, then the rat EDL muscle should be very sensitive to such a change when creatine phosphate levels are low. In an attempt to test this prediction and to gain more information about the physiological role of creatine phosphate in skeletal muscle contraction the following questions were asked:

1. Is the response following brief bursts of activity in rat EDL muscle affected by creatine phosphate depletion?

2. Which contractile parameters of the rat EDL are most affected by creatine phosphate depletion?

3. What possible mechanisms may link the observed changes in contractile parameters with the depletion of creatine phosphate?

METHODS

There are two distinct parts to this investigation, separated primarily on the basis of the differing protocol followed and the different apparatus used in the contraction studies. The animals obtained for these two parts are treated as separate groups for the biochemical analyses as well (Part I and Part II).

TREATMENT OF ANIMALS

1) Rats on the analogue diet from 5 days to 50 days of age

In a preliminary feeding trial, four complete litters of Sprague-Dawley rats containing an even distribution of male and female pups were purchased from Charles River Canada Incorporated in Montreal within a day or so after the birth of the litter. Pups in each litter were randomly separated into three groups: 1. control, 2. analogue-fed from weaning (23 days old) to 50 days old, and 3. analogue-fed from 5 days to 50 days old. At 5 days of age pups from groups 1 and 2 were administered distilled water by intubation and pups from group 3 received, also by intubation, distilled water containing 1% of β-guanidinopropionic acid (GP) obtained from Sigma Chemical Co. Each pup received 10 µl of fluid per gram body weight per day and this continued to weaning. The smallest volume possible was used so as not to interfere with suckling.

At weaning pups were separated from their mother, placed in individual cages, and those from group 1 were given 10 grams of powdered Purina Rat Chow per day while those from group 2 and 3 received 10 grams of powdered Chow containing 1% of GP per day. This quantity was adjusted to maintain a steady growth rate so that by 50 days of age rats were eating 12 grams of Chow per day. Water was provided *ad libitum*. The dosage level for both intubation and chow feeding corresponds to approximately 1 mg of GP per gram body weight per day.

This experiment was done in an attempt to further reduce muscle creatine levels from those achieved by analogue feeding from weaning.

2) Rats on the analogue diet from weaning (21 days old) to 50 days old.

Male weanling rats purchased from Charles River Canada Incorporated comprised the two groups of animals used in Parts I and II of this study. Rats were randomly assigned to control and treated groups and were individually caged. Control rats were fed powdered Chow and treated rats powdered Chow containing 1% GP in the quantities and dosage level described above. The GP used in Part I was purchased from Sigma Chemical Co. and in Part II it was synthesized in our laboratory from β-alanine and cyanimide according to the method of Rowley *et al.* (1971). There were virtually no chemical differences between the purchased and synthesized analogues that we could detect from either NMR spectra or from melting point depression analysis and we assumed that any impurities present were minor.

BIOCHEMICAL TECHNIQUES

1. Freezing and extraction

EDL muscles of four normal and four GP-fed rats from both Part I and II were used for biochemical analysis. After the rat reached the appropriate depth of anaesthesia, the EDL muscles from both legs were denervated, quickly excised and frozen between aluminum blocks precooled in liquid nitrogen. A styrofoam weighing dish with a lid was used to obtain muscle weights while keeping the muscles frozen. The range of muscle weights was between 50 and 80 mg. Frozen muscles were then placed in small, cold plastic tubes and stored at -70°C for later extraction.

An aluminum block with holes to fit the small tubes was precooled in liquid nitrogen to keep the muscles cold during extraction. A stainless steel rod shaped to fit the tubes, also cooled in liquid nitrogen, was used to grind the muscles down to a fine powder. The tubes were placed on ice, 0.5 ml of 6% perchloric acid was added, the contents were vortexed, and the mixture was allowed to stand on ice for 20 minutes. Following a second vortex and centrifugation at 10,000g for 10 minutes to precipitate the protein, the supernatant was decanted into a cold fresh tube and neutralized with 100 µl of 2.5 M K_2CO_3. Centrifugation for 10 minutes at 10,000g caused the potassium perchlorate to precipitate out and the supernatant was pipetted into a cold fresh tube. The extracted sample was either frozen immediately and stored at -70°C or used right away for analysis.

2. Acid hydrolysis

For the determination of total creatine (creatine + creatine phosphate), 120 µl of 0.5N HCl was added to 0.5 ml of the sample and the mixture was heated to 84°C for 10 minutes to hydrolyze all the creatine phosphate present. Immediately following heating, the samples were placed on ice and neutralized with 12 µl of 2.5M K_2CO_3.

3. Determination of total creatine

A modification of the colourimetric assay described by Ennor and Rosenberg (1952) was used for the determination of total creatine content in hydrolyzed muscle extracts. The reaction mixture contains 1.0 ml distilled water, 0.1 ml muscle extract, 0.5 ml of 1% α-naphthol dissolved in a concentrated alkali solution, and 0.05 ml of a 1% solution of butane-2,3-dione diluted 1:20 in distilled water. After letting the reaction mixture sit for 30 minutes it is read spectrophotometrically at 510 nm at room temperature.

From previous reports in the literature, samples were expected to contain approximately 25 μmoles of creatine per gram wet weight of muscle tissue. The standard curve was made to range from 0 to 100 nmoles.

4. Determination of total β-guanidinopropionic acid

A colourimetric assay known as the Sakaguchi colour reaction (Sakaguchi, 1925) was employed for the estimation of guanidino compounds. This assay has been described more recently by Bonas *et al.* (1963). The reaction mixture contains 1.0 ml distilled water, 0.1 ml of hydrolyzed muscle extract, 0.25 ml of a thymine-α-naphthol mixture, 0.1 ml NaOCl, and 0.1 ml of 2% thiosulfate. The mixture is allowed to stand on ice for 5 minutes and is read spectrophotometrically at 510 nm at 10°C.

5. Determination of ATP and CrP

A combined enzymatic assay is used for the estimation of ATP and CrP content in unhydrolyzed muscle extracts as described by Lamprecht and Stein (1965). With this procedure a sequential series of three reactions occurs:

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightleftharpoons{1} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

$$\text{Glucose} + \text{ATP} \xrightleftharpoons{2} \text{Glucose-6-phosphate} + \text{ADP}$$

$$\text{Creatine phosphate} + \text{ADP} \xrightleftharpoons{3} \text{Creatine} + \text{ATP}$$

where 1 = glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 2 = hexokinase (EC 2.7.1.1), and 3 = creatine kinase (EC 2.7.3.2). Reaction 1 is completed first to remove any endogenous glucose-6-phosphate. Reaction 2 then removes endogenous ATP so that reaction 3 can measure creatine phosphate content in the absence of ATP. The ATP content and the creatine phosphate content in reactions 2 and 3, respectively, is measured directly by the increase in optical density at 340 nm due to the formation of NADPH.

6. Corrections and analysis

Since GP reacts to some extent with butane-2,3-dione as well, it is necessary to correct for this in the estimated creatine content. A known amount of GP was added to the reaction mixture in the absence of creatine and read as described above for the creatine assay. The correction factor (F) was obtained from the ratio of this GP reading to the Cr reading from an equivalent amount of creatine. The corrected creatine reading was then obtained from the following formula:

$$Q_C = Q_{Cr} - F \times Q_{GP}$$

where Q_C = the corrected creatine value, Q_{Cr} = the creatine reading from the standard curve, F = the correction factor, and Q_{GP} = the GP reading from the GP standard curve.

For the estimation of the free energy change of ATP hydrolysis ($-dG/d\xi$) the following equation from Dawson *et al.* (1980) was used:

$$-dG/d\xi = \Delta G^{o}_{obs} + RT\ \ln([Cr][P_i]/K[CrP][H^+])$$

where $\Delta G^{o}_{obs} = 36$ kJmol^{-1}, $[P_i] = 4\times10^{-3}$ M, $K = 2\times10^{9}$ M^{-1}, and $[H^+] = 10^{-7}$M. Other assumptions involved in the use of this equation are that the Lohmann reaction is at equilibrium, that the formation of phosphorylated GP in creatine depleted muscle does not significantly affect the equilibrium position, and that the equilibrium concentrations of the substrates provide a useful estimate of the substrate concentration during muscular activity.

For the estimation of cross-sectional area we first calculated muscle volume (mass/density) assuming muscle density to be 1.05×10^{3} mg.cm^{-3} (Hill, 1965). This value was then divided by muscle length. We assumed cross-sectional area to be constant along the length of the muscle.

All values obtained from the biochemical assays are expressed as the mean ± the standard error of the mean. Statistical t-tests were performed on this data and the probability level $p<0.05$ was chosen for significance.

CONTRACTION STUDIES

1. Surgical procedure

Rats were anaesthetized with 65 mg of sodium pentobarbitol per kg body weight, administered intraperitoneally. The EDL muscle was carefully isolated from surrounding tissues leaving its nerve and blood supply intact. To avoid reflex activity the common peroneal nerve was cut about 1 cm distal to the point at which the deep peroneal nerve branch crosses the fibula. The proximal tendon and the ankle joint were clamped to stay firmly in position and the distal tendon was cut close to its point of insertion, tied to a thin steel wire with silk thread, and connected to a force transducer.

The rat was immersed in a bath containing Tyrode's solution with the following concentration of ions: 145.5 mM Na^{+}, 2.5 mM Ca^{2+}, 4.7 mM K^{+}, 3.1 mM Mg^{2+}, 129.0 mM Cl^{-}, 25.0 mM HCO_3^{-}, 1.2 mM $H_2PO_4^{2-}$, 3.1 mM SO_4^{2-}, and 5.5 mM glucose. Solution temperature was maintained between 35° and 36° celsius by a circulating water jacket and the solution was gassed with 95% oxygen and 5% carbon dioxide.

2. Electrical system

Muscles were made to contract by direct field stimulation with a Grass S88 stimulator using platinum strip electrodes placed 1-2 mm on either side. Length was adjusted so that peak twitch tension was maximal. Isometric tension development was converted to an electrical output by four strain gauges which formed a wheatstone bridge. The voltage output was amplified and differentiated then photographically recorded by a Tektronix 502A Dual Beam Oscilloscope with a camera attachment for later analysis. The differentiating circuit, calibrated using triangular wave pulses generated by a Hewlett Packard 3300A Function Generator, had a time constant of 120 µsec.

3) Stimulation Patterns

PART I

To measure isometric tension development a Statham Universal Transducing Cell (Model UC2) was used with a load cell attached to extend its range beyond 200 grams. The compliance of the transducer, load cell, plus the steel wire was 4×10^{-3} cm/g and two distinct resonant frequencies of 30 and 250 cycles per second were measured.

1) Repeated trains of pulses

Mainwood *et al.* (1982b) previously used isolated diaphragm strips and delivered tetanic trains at 2 per second. In this fast twitch EDL muscle preparation preliminary experiments showed that the shorter stimulus interval from a 3 per second series resulted in a more pronounced effect on the tetanic contraction parameters we were measuring.

Using a Devices digitimer Type 3290 to set the cycle length and to trigger the stimulator, oscilloscope and camera at appropriate times, 100 millisecond (msec) tetanic trains were delivered at 3 per second for 20 seconds and every other contraction was photographically recorded. A stimulation frequency of 140-180 Hertz was required for a smooth fused tension recording.

ii) Variable duration of single conditioning tetanus

As a follow up to the series stimulation a single conditioning tetanus of variable duration was delivered followed by a single 100 msec test tetanus. The length of the conditioning tetanus was controlled by the digitimer and the durations used were 0.05, 0.2, 0.7, 1.0, and 1.5 seconds. The 100 msec test tetanus was delivered 230 msec later which corresponds to the interval between each of the tetani in the 3 per second series. A 5 minute rest period was allowed before the next conditioning stimulus was delivered.

iii) Variable interval between conditioning and test tetanus

Once the optimal duration of the conditioning stimulus was established (1 second), the time course of its effect on the subsequent test tetanus was examined. A single test tetanus was triggered after each 1 second conditioning tetanus and a five minute rest was allowed in between. Intervals of 0.05, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, and 2.0 seconds between the conditioning and test tetani were chosen.

PART II

A Kulite strain gauge Type BG±100 grams (Durham Instruments) was used for this section. The voltage output was again linear to more than 200 grams. The compliance of the transducer is about 1.8×10^{-5} cm/g and the natural resonant frequency of the transducer plus the steel wire was measured at about 1.7 kHz. This corresponds to a fractional muscle shortening of about 0.01% and a transducer response time of less than 0.1 msec. A rest interval of 5 minutes was used throughout this section.

i) Single twitch before and after conditioning tetanus

In this part of the study, posttetanic twitch responses were examined. It was felt the single twitch may be a more sensitive indicator of the differences existing between normal and depleted muscles. A twitch contraction was delivered immediately before (100 msec) a 1 second conditioning tetanus and again 200 msec after. The twitch after was compared to the twitch before the conditioning tetanus in an attempt to isolate the effects of the conditioning stimulus from other unaccounted for effects, (*e.g.*, time).

ii) Variable interval between tetanus and second twitch

The time course of the posttetanic effects was examined by varying the time interval (t_2) between the tetanus and the second twitch. A single twitch was triggered after each 1 second tetanus and an interval of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 60, or 120 seconds was chosen. The first twitch was delivered 100 msec before the tetanus as described previously.

iii) Variable duration of conditioning tetanus

Observations from the time course experiments led to questions concerning the effect of varied tetanic duration on posttetanic responses. Durations of 0.1, 0.2, 0.4, 0.5, 0.6, 1.0, 1.5 and 2.0 seconds were used and twitch contractions were delivered 100 msec before and 200 msec after.

iv) Multiple pulses before and after conditioning tetanus

Since the mechanisms behind the differing posttetanic responses in normal and creatine depleted muscles were still unclear, we attempted to examine this further by looking at the effect of the conditioning stimulus on multiple pulse responses. 1, 2, 3 and 4 pulses were delivered, each before and after the 1 second conditioning tetanus as described above. The multiple pulses were 2.0 msec apart as this interval was found to be optimal in preliminary experiments.

4) Corrections and analysis

The photographic records obtained from all experiments contain tracings of tension and its derivative. Measurements of peak tension (T), the maximum rate of tension development ($+\dot{T}$), and the maximum rate of relaxation ($-\dot{T}$) were made using a photographic enlarger. The amplitude of these curves was measured in cm and was converted to the corresponding voltage value by a multiplication factor obtained from previous calibrations of oscilloscope grid photographs.

The final values obtained in N or $Nsec^{-1}$ were statistically manipulated and are expressed as mean values ± the standard error of the mean for each experiment. To test for significance, t-tests were performed and the probability level $p < .05$ was chosen.

RESULTS

PRELIMINARY FEEDING TRIAL

Eight rats from the group fed 1 mg GP/g body weight/day, four from 5 days to 50 days old and four from 23 days (weaning) to 50 days old, were used for muscle biochemistry. The total creatine content (creatine + creatine phosphate) of the extensor digitorum longus (EDL) muscles from the two groups is given in Table 1. Control values are included for comparison. This preliminary experiment shows that feeding the analogue shortly after birth does not deplete muscle creatine any further than feeding GP from weaning.

The effect of the two analogue feeding techniques on body weight and EDL muscle weight is given in Table 2. The mean body weight of rats fed GP from 5 days old is significantly different from that of control rats: 124.6 ± 3.4 g and 141.6 ± 3.4 g for GP-fed and control, respectively, but there is no difference between body weights of control rats and rats fed GP from weaning. The mean EDL muscle weights show evidence of growth retardation in animals from both GP-fed groups.

Growth curves of animals on this feeding technique from both 5 days old and from weaning are displayed in Figures 2 and 3, respectively. In Figure 2, total body growth of control and treated rats ~~is~~ the same until weaning. At weaning (23 days old)

Table 1. Effect of age at the start of analogue (BP) feeding on total creatine content (creatine + creatine phosphate) of frozen, resting denervated EDL muscles (n=4).

	CONTROL	FED FROM 5-50 D.O.	FED FROM 21-50 D.O.
TOTAL CREATINE*	23.0 ± 1.4	4.1 ± 0.6	4.5 ± 0.3

* values are means ± standard error of the mean

units are $\mu mol g^{-1}$ wet muscle weight

Table 2. Comparison of body weight (in grams) between control and GP-fed rats at 49 days of age and EDL muscle weight (in milligrams) between control and GP-fed rats at approximately 50 days of age. The numbers in brackets denote sample size.

	BODY WEIGHT		UNSTIMULATED EDL WEIGHT	
	5 D.O.	weaning	5 D.O.	weaning
CONTROL	141.6±3.4** (10)	136.3±1.4 (10)	70.5±2.9** (8)	76.4±1.6** (9)
DEPLETED	124.6±3.4* (10)	131.5±2.0 (10)	53.6±2.1* (9)	69.7±1.4* (10)

* significant difference between animals fed GP from 5 days old and from weaning; $p<.05$

** significant difference between control and depleted animals; $p<.05$

Figure 2. Growth curves of newborn rats fed distilled water (control: closed circles) or 1% GP (treated: open circles) by intubation from 5 days old to weaning, then ground chow (control) or chow containing 1% GP (treated) from weaning to 50 days of age. (n=10 except where otherwise indicated). The arrow shows the age of the animals at weaning.

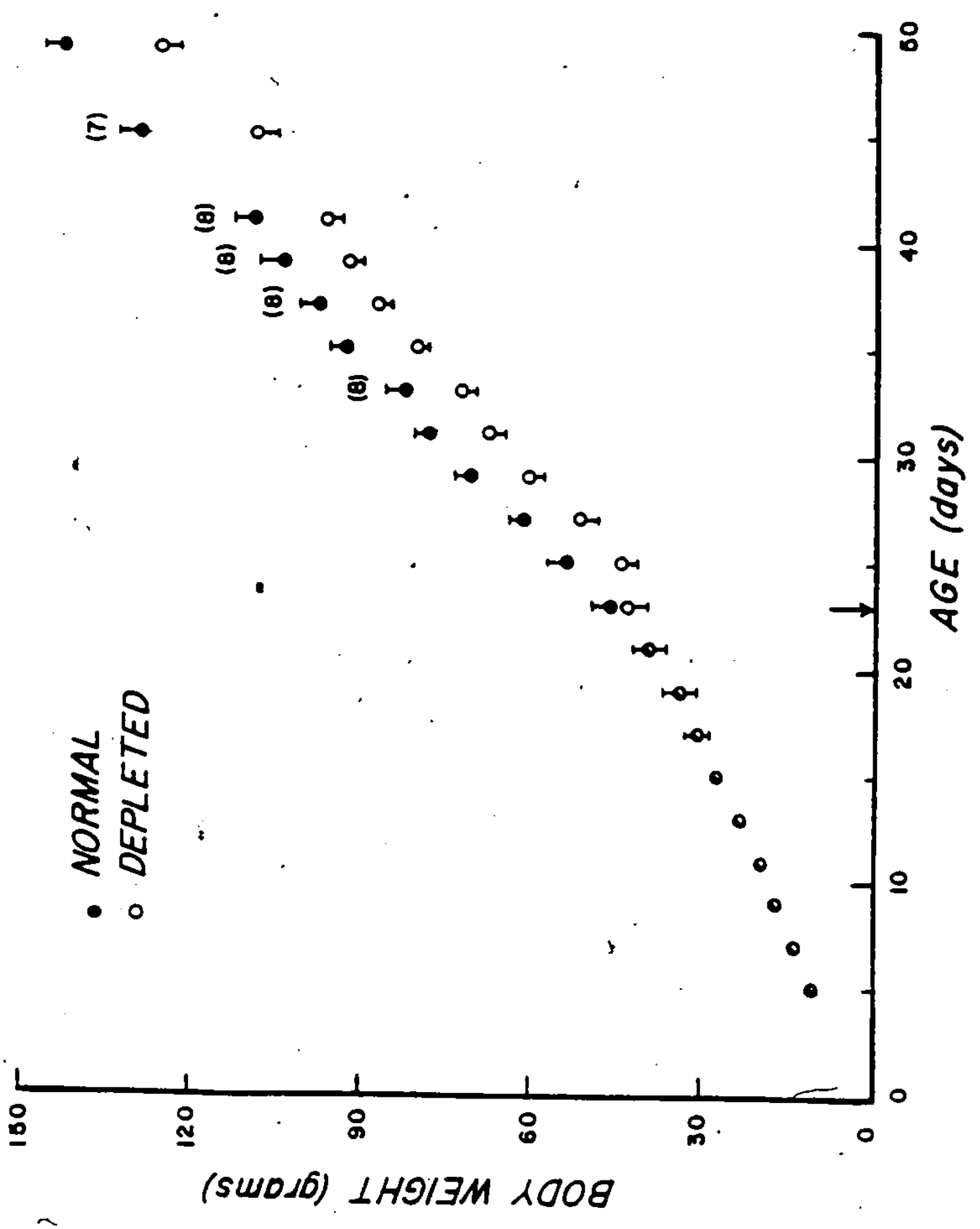
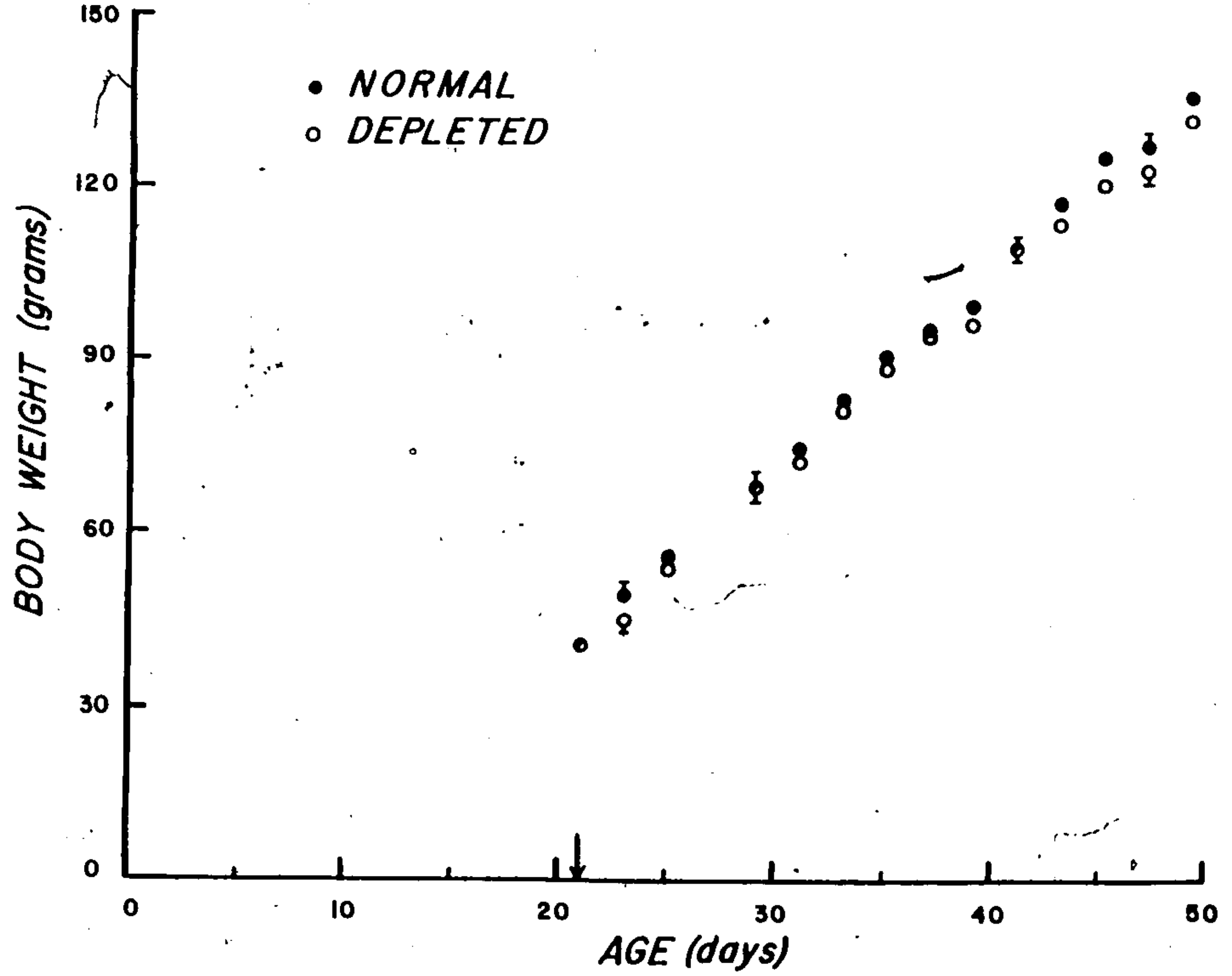

● NORMAL
○ DEPLETED
BODY WEIGHT (grams)
AGE (days)
0
30
60
90
120
150
0
10
20
30
40
50
(8)
(8)
(8)
(8)
(7)

Figure 3. Growth curves of rats fed ground chow (control; filled circles) or chow containing 1% GP (open circles) from weaning to 50 days old. (n=10). The arrow shows the age of the animals at weaning.


● NORMAL
○ DEPLETED
BODY WEIGHT (grams)
150
120
90
60
30
0
0
10
20
30
40
50
AGE (days)

the mean body weight of rats on the GP diet diverges from and remains below that of normal rats. Total body growth of rats on the GP diet from weaning only is not different from normal (Figure 3).

BIOCHEMISTRY

Biochemical analysis of EDL muscle extracts from 50 day old rats revealed that creatine (Cr) and creatine phosphate (CrP) levels were reduced in the animals fed 1% GP from weaning. Results from Part I (treated animals fed the GP from Sigma) and Part II (treated animals fed our laboratory synthesized GP) are given in Tables 3 and 4, respectively. Total muscle Cr in both groups of GP-fed animals fell by about 80%. The mean total Cr content in Part II however, is about 30% greater than in Part I and the total GP content is about twice as high. CrP levels consistently fell by about 96% and ATP content was reduced by 64% and 79% in Part I and II, respectively. Although the CrP/Cr ratios in normal muscles from Parts I and II are different (1.6 ± 0.3 *vs* 0.6 ± 0.04), in both cases they are around ten times greater in normal than in depleted muscles.

The calculated free energy change ($-dG/d\xi$) for ATP hydrolysis shows a decrease of about 10% in both GP-fed groups of animals.

Resting depleted muscles therefore, have a reduced high

Table 3. Biochemical analysis of resting denervated EDL muscles excised from 50 day old normal rats and rats fed 1% GP from weaning. (GP was purchased from Sigma)

	CONTROL	DEPLETED
n	4	4
Total Cr*	23.0 ± 1.4	4.5 ± 0.3
Total GP*	0.3 ± 0.2	13.4 ± 1.2
CrP*	13.8 ± 1.8	0.5 ± 0.03
ATP*	5.6 ± 0.5	2.0 ± 0.2
CrP/Cr	1.6 ± 0.3	0.1 ± 0.02
$-dG/d\xi$**	66.7	59.9

* in $\mu mol \cdot g^{-1}$ wet weight; as mean ± standard error of the mean

** in $kJ \cdot mol^{-1}$

Table 4. Biochemical analysis of resting denervated EDL muscles excised from 50 day old normal rats and rats fed 1% GP from weaning. (GP was synthesized in our laboratory)

	CONTROL	TREATED
n	4	4
Total Cr*	30.6 ± 0.4	5.9 ± 0.3
Total GP*	0.4 ± 0.2	29.5 ± 1.1
CrP*	11.6 ± 0.6	0.5 ± 0.2
ATP*	6.3 ± 0.1	1.3 ± 0.1
CrP/Cr	0.62 ± 0.04	0.09 ± 0.02
$-dG/d\xi$ **	64.4	59.5

* in $\mu mol \cdot g^{-1}$ wet weight; as mean ± standard error of the mean

** in $kJmol^{-1}$

energy phosphate pool (ATP + CrP), a reduced free energy change for ATP hydrolysis, and an altered CrP/Cr ratio. These striking biochemical changes are accompanied by alterations in various contractile parameters as shown in the following section.

CONTRACTION STUDIES - PART I

1) Effects of creatine depletion on a single twitch and tetanus

Representative examples of twitch and tetanic contraction characteristics obtained from normal and creatine depleted EDL muscles are displayed in Figure 4. Tension (T), the maximum rate of tension development ($+\dot{T}$), and the maximum rate of relaxation ($-\dot{T}$), were measured from traces such as these and the results of their analysis are given in Table 5. Although the mean twitch tension in depleted muscle is about 20% less than normal, this difference is not significant. Time to peak isometric twitch tension was measured in 4 normal and 5 depleted rats. The mean values ± SEM are 15.1 ± 0.7 and 15.6 ± 0.5 msec, respectively. Thus, twitch characteristics are unchanged by creatine depletion while tetanic tension is reduced by 35% and tetanic $+\dot{T}$ and $-\dot{T}$ are reduced from normal by 35% and 51%, respectively.

Since depleted muscles are smaller than normal, a different picture emerges upon examination of the normalized values. The differences between control and treated T and $+\dot{T}$ are no longer

Figure 4. Isometric twitch (a and c) and tetanic (b and d) tension recordings from normal (left) and creatine depleted (right) EDL muscle. In each case the lower trace is tension in newtons (N) and the upper trace is the derivative of tension in newtons per second ($Nsec^{-1}$). For the tetanus 16 pulses at 500 Hertz were delivered.

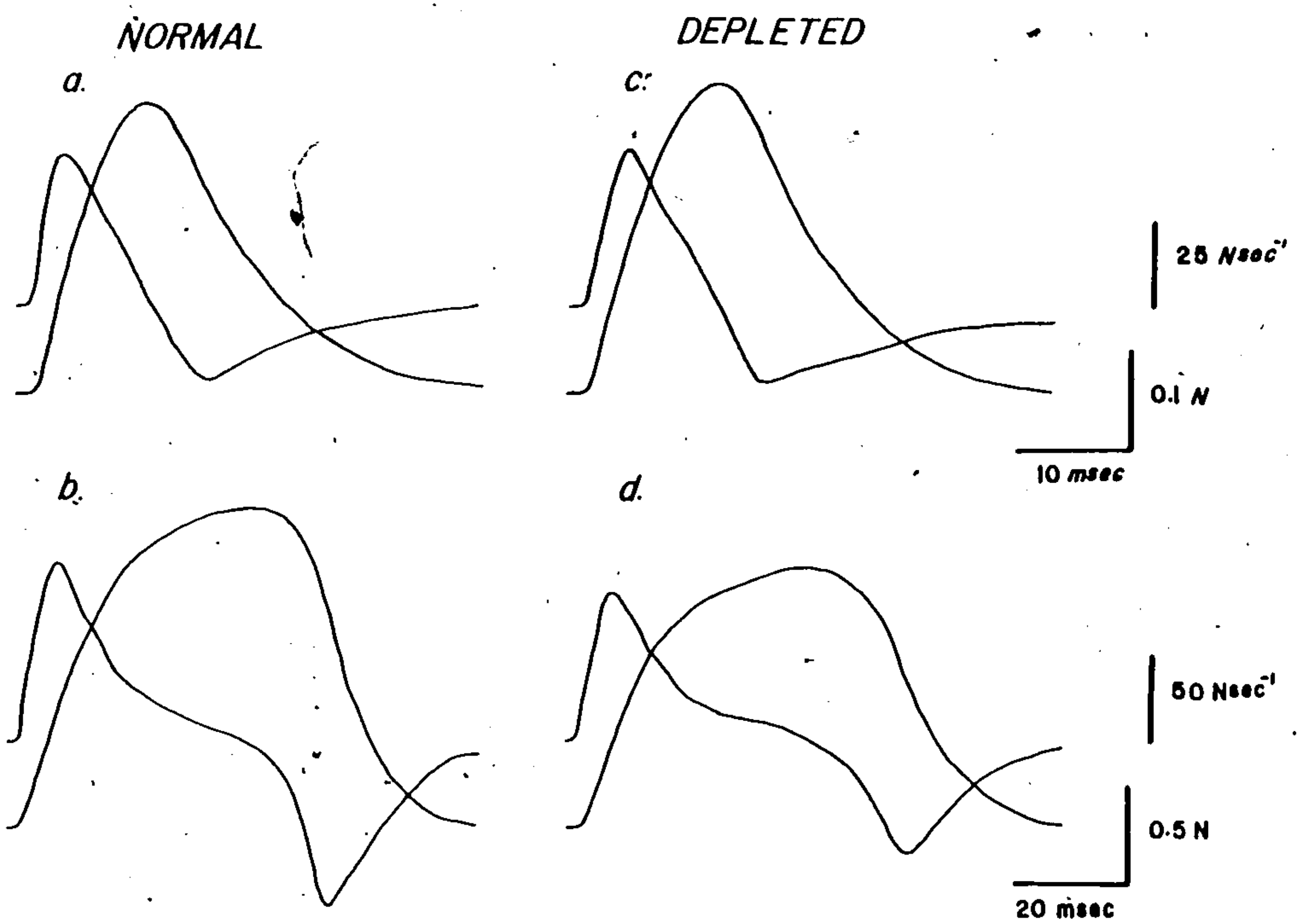

NORMAL
DEPLETED
a.
c.
25 Nsec⁻¹
0.1 N
10 msec
b.
d.
50 Nsec⁻¹
0.5 N
20 msec

Table 5. Contractile parameters of a single twitch and a single tetanus in the EDL muscle of control (n=6) and creatine depleted (n=7) 50 day old rats.

		NORMAL x ± SEM	DEPLETED x ± SEM	
TWITCH	T	257.5 ± 19.5 mN	207.2 ± 18.0 mN	
	$+\dot{T}$	25.2 ± 2.0 Ns^{-1}	22.3 ± 2.6 Ns^{-1}	
	$-\dot{T}$	13.3 ± 1.1 Ns^{-1}	11.9 ± 1.0 Ns^{-1}	
NORMALIZED TWITCH	T/CSA	11.9 ± 1.2	12.4 ± 1.7[A]	
	$+\dot{T}$/CSA	1143.3 ± 110.4	1324.3 ± 183.7[B]	
	$-\dot{T}$/CSA	603.2 ± 63.1	702.2 ± 66.0[B]	
TETANUS	T	1.7 ± 0.1 N	1.1 ± 0.05 N	*
	$+\dot{T}$	67.4 ± 5.8 Ns^{-1}	44.0 ± 3.2 Ns^{-1}	*
	$-\dot{T}$	97.5 ± 7.5 Ns^{-1}	49.9 ± 4.2 Ns^{-1}	*
NORMALIZED TETANUS	T/CSA	78.9 ± 7.5	66.0 ± 8.3[A]	
	$+\dot{T}$/CSA	3065.2 ± 336.7	2632.7 ± 327.0[B]	
	$-\dot{T}$/CSA	4107.9 ± 277.6	2975.6 ± 367.8[B]	*

[A] in Ncm^{-2}

[B] in $Ns^{-1}cm^{-2}$

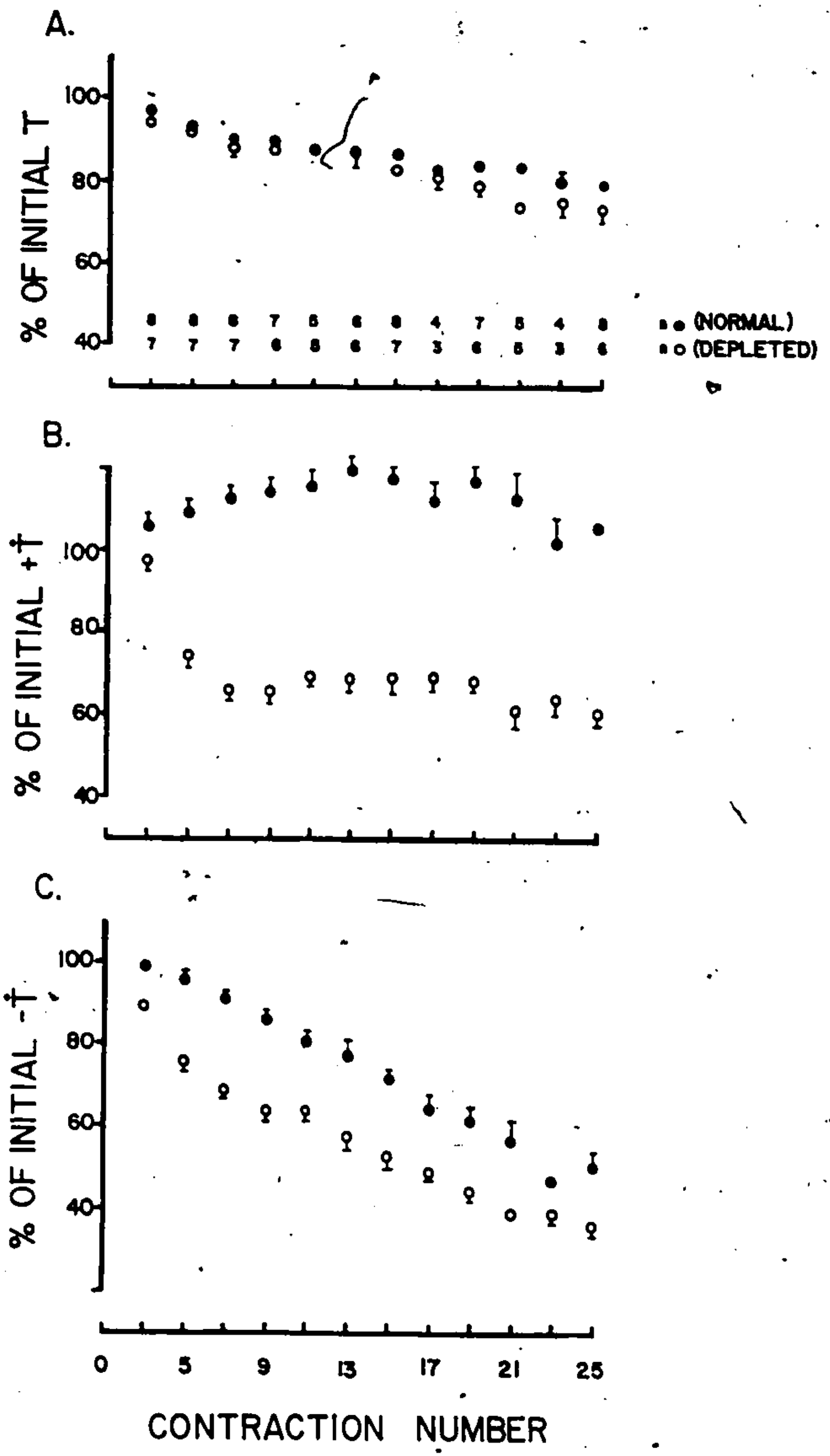
* $p < .05$

significant whereas $-\dot{T}$ is still significantly less when expressed as a function of the estimated cross-sectional area. It appears then that, during a tetanus, $-\dot{T}$ is the parameter most affected by creatine depletion.

2) Series stimulation

It was previously observed by Mainwood *et al.* (1982b) with isolated rat diaphragm strips, that after 3 or 4 tetanic contractions in series (2 per sec) the maximum rate of tension development ($+\dot{T}$) decreased in creatine depleted muscles while it increased in normal muscles. This pattern of stimulation was repeated with our *in situ* EDL muscle preparations. A series of 100 msec tetanic stimuli (140-180 Hertz) was delivered at a rate of 2 per second. Percent of initial T, $+\dot{T}$, and $-\dot{T}$ is plotted on the ordinate of the three graphs and contraction number on the abcissa in Figure 5. 100% represents the initial value for a single 100 msec tetanus in the beginning of the series in all three cases. Points after this show changes during the series. Both T and $-\dot{T}$ gradually decline in normal and depleted muscles as the number of contractions increases. The decrease in T is slightly less in normal muscles; by contraction number 25, normal muscles develop 78.7 ± 2.0% and depleted muscles 72.0 ± 1.6% of the initial tension. Depleted muscles show an initial rapid decline in $-\dot{T}$ which levels off to approach 34.7 ± 1.3% while normal muscles show a steady decline to approach 49.3 ± 3.5% of the initial $-\dot{T}$ by the 25th contraction.

Figure 5. Effects of a series of tetanic stimuli (140-180 Hertz) 100 msec in duration, delivered at 3 per second, on tension (T), the maximum rate of tension development ($+\dot{T}$), and the maximum rate of relaxation ($-\dot{T}$) in normal (filled circles) and creatine depleted (open circles) EDL muscles. Contraction number is plotted on the abcissa and percent of the initial T (A), $+\dot{T}$ (B) or $-\dot{T}$ (C) is shown along the ordinate. The two rows of numbers above the abcissa in 5A refer to the sample size of normal (top) and depleted (bottom) animals and are the same in B and C.


A.
% OF INITIAL T
100
80
60
40
8 8 8 7 5 6 8 4 7 5 4 8
7 7 7 6 5 6 7 3 6 5 3 6
n ● (NORMAL)
n ○ (DEPLETED)
B.
% OF INITIAL +Ṫ
100
80
60
40
C.
% OF INITIAL -Ṫ
100
80
60
40
0
5
9
13
17
21
25
CONTRACTION NUMBER

A much more striking difference between the two muscle groups was observed in $+\dot{T}$: it is potentiated in normal muscles and rapidly declines in depleted muscles during the series of tetanic stimuli. Thus the effect initially observed with *in vitro* diaphragm strips has been confirmed using an *in situ* EDL preparation.

3) Variable duration of a single conditioning tetanus

After a total stimulation time of about 1 second a maximum effect on $+\dot{T}$ seems to be reached for both normal (about 10% potentiation) and depleted (about 30% depression) muscles. This response could be associated with the number of contraction-relaxation cycles or it could be a function of the total contraction time. To examine this further we looked at changes in $+\dot{T}$ following a single conditioning tetanus of varying duration. Figure 6 confirms that the maximum effect on $+\dot{T}$ of the test tetanus in both normal and depleted muscles occurs when the conditioning tetanus is 1 second in duration.

A representative example of the tension recordings from this stimulation procedure is shown in Figure 7. The traces on the left show the tension developed and its derivative during a single tetanus in a normal (top) and a creatine depleted (bottom) muscle. The end of the 1 second conditioning tetanus and the 100 msec test tetanus are shown on the right. A 230 msec interval separates the conditioning from the test tetanus - the

Figure 6. Effect of varying the duration of the conditioning tetanus on the maximum rate of tension development (+$\dot{T}$) in normal (filled circles) and creatine depleted (open circles) EDL muscles. The duration was varied from 50 msec to 1.5 seconds. Percent of the initial maximum rate of tension development (% of initial +$\dot{T}$) is plotted on the ordinate and duration is in seconds along the abcissa. (n=3 in all cases)

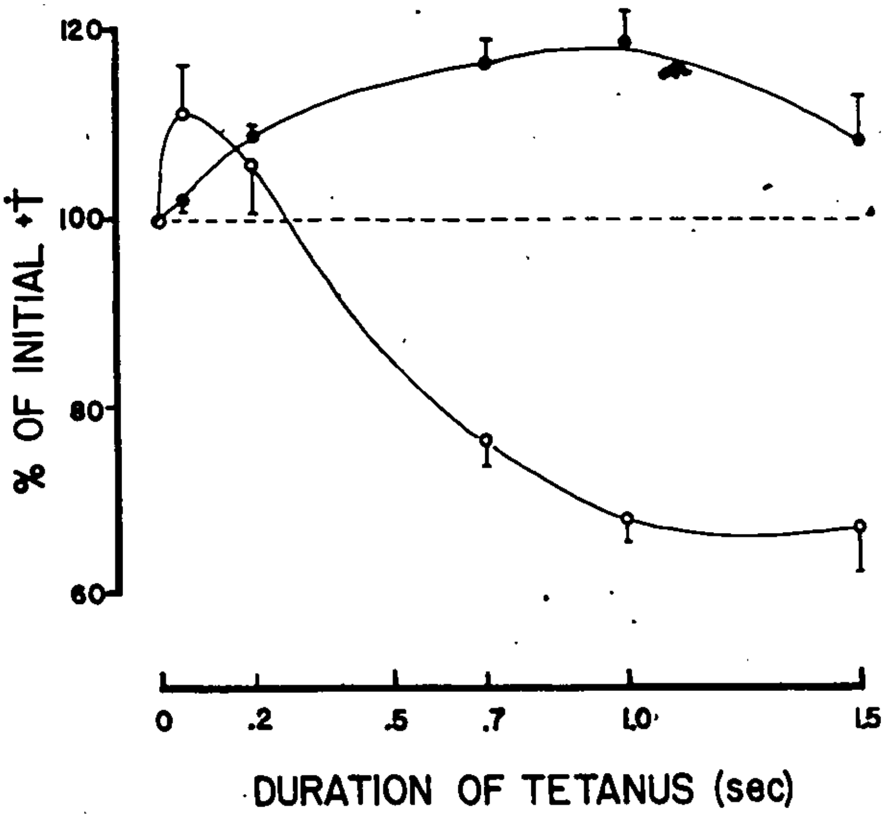
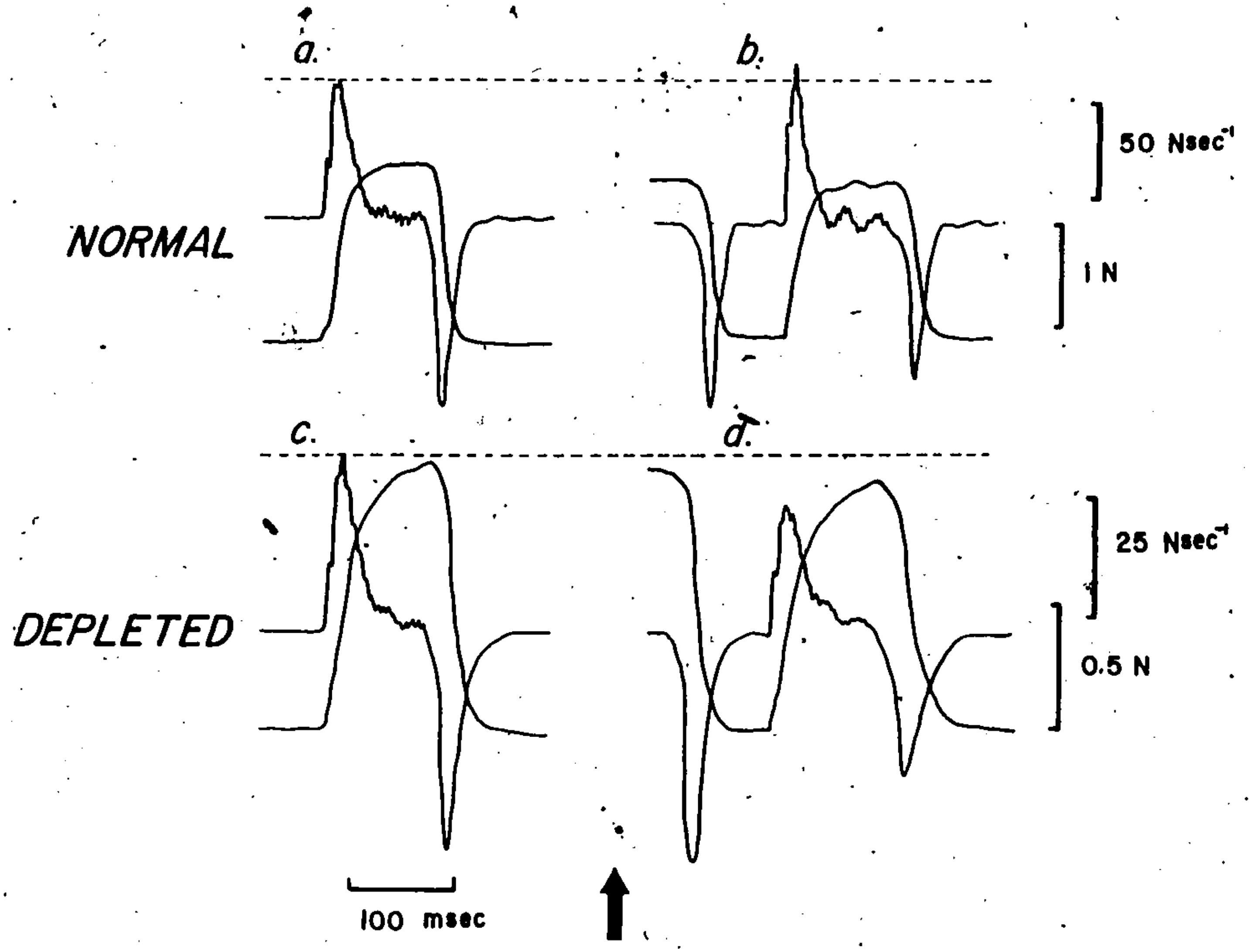

120
100
80
60
% OF INITIAL +T
0
.2
.5
.7
1.0
1.5
DURATION OF TETANUS (sec)

Figure 7. Isometric tension recordings of 100 msec 160 Hertz tetanic contractions before (left) and 230 msec after (right) a 1 second conditioning tetanus (arrow) in normal (a and b) and creatine depleted (c and d) muscles. In each case the lower trace is tension in newtons (N) and the upper trace is the derivative of tension in newtons per second ($Nsec^{-1}$). The dotted lines drawn across the tracings emphasize the change in the maximum rate of tension development ($+\dot{T}$) in both muscle groups following the conditioning stimulus.


a.
b.
NORMAL
50 Nsec^{-1}
1 N
c.
d.
DEPLETED
25 Nsec^{-1}
0.5 N
100 msec

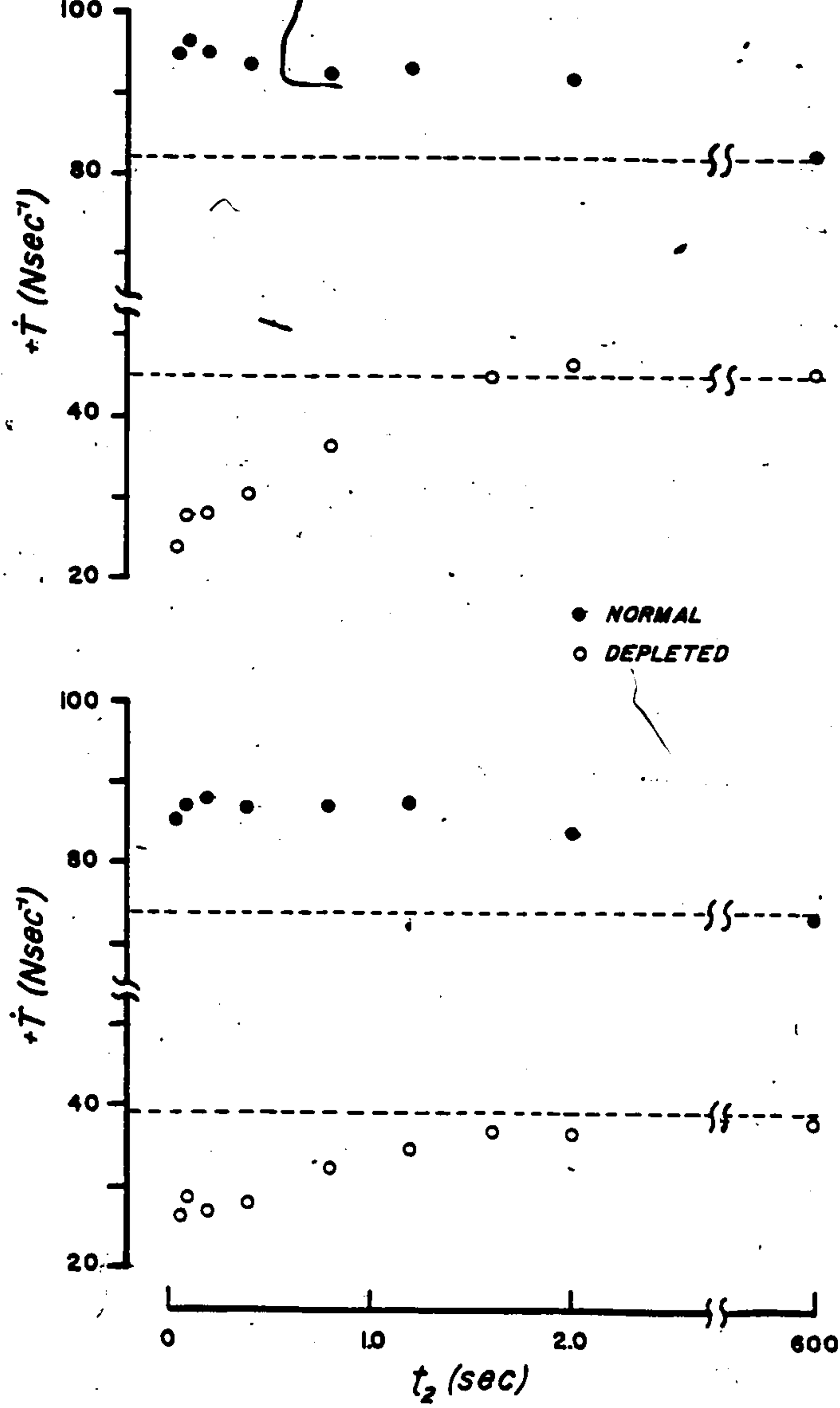
time interval between each of the tetani in the 3 per second series. Again, $+\dot{T}$ is increased in normal muscles by about 10% and decreased in depleted muscles by about 30%. These changes are emphasized by the dotted lines extending from $+\dot{T}$ of the single tetanus to that of the test tetanus. The extent of potentiation and depression appears to be similar following either a single 1 second tetanus or ten 100 msec tetani.

4) Variable interval between conditioning and test tetanus

To determine the time course of the potentiation and the depression a 1 second conditioning tetanic train was delivered as before, but the interval between it and the subsequent test tetanus (t_2) was varied. Figure 8 shows the results from two normal and two depleted muscles. Normal muscles show a potentiation of $+\dot{T}$ which gradually declines as the interval (t_2) increases and the depression seen in depleted muscles is rapidly reversed to pre-conditioning levels within seconds. At 2 minutes the potentiation has almost disappeared. This difference in time course suggests two different mechanisms are in effect.

Upon closer inspection of the traces in Figure 7, one can see that the derivative curves appear bumpy. This raises the question of whether there is incomplete fusion of the tetanus or whether this is due to resonance of the measuring system. The Statham transducer plus the apparatus attached to it did exhibit a low resonance frequency (about 30 cps). Yet the frequency of

Figure 8. Time course of the effects of a 1 second conditioning tetanus on the maximum rate of tension development (+$\dot{T}$) of a subsequent 100 msec test tetanus (140-180 Hertz) in normal (filled circles) and creatine depleted (open circles) muscles. The interval between the conditioning and the test tetanus, t_2, is plotted on the abcissa in seconds, and the maximum rate of tension development (+$\dot{T}$) in Nsec^{-1} is plotted on the ordinate. Each series of points represents one animal. The dotted lines indicate the maximum rate of tension development during a preconditioning 100 msec tetanus.



100
80
40
20
$+\dot{T}$ (Nsec^{-1})
● NORMAL
○ DEPLETED
100
80
40
20
$+\dot{T}$ (Nsec^{-1})
0
1.0
2.0
600
t_2 (sec)

these bumps seems to coincide with the tetanic stimulus interval of 6.25 msec (160 Hertz). Additionally, it can be very clearly seen that the very first bump in the normal +I curve is greater in amplitude following the 1 second conditioning stimulus than for a single tetanus. In depleted muscles this first bump looks smaller.

These considerations suggested the need for a better transducer as well as the idea of investigating the effects of the conditioning tetanus on a single twitch response. Thus in Part II we used the more sensitive Kulite strain gauge and looked at posttetanic twitch responses.

Part II

A preliminary series of experiments were performed in which we varied stimulation frequency with the new transducer. In order to get a smooth response and a maximum rate of tension development, we needed a frequency of 300 - 400 Hertz. Thus, intervals of less than about 3 msec between pulses are required to fuse the twitch responses and obtain maximum activation.

1) Single twitch before and after a tetanus

Twitch records were obtained both 100 msec before and 200 msec after the 1 second conditioning tetanus from normal and creatine depleted muscles. The data is given in Table 6. Again,

Table 6. Comparison of twitch contraction parameters before and after a 1 second tetanus from normal and creatine depleted EDL muscles from 50 day old rats.

			BEFORE	AFTER	% CHANGE
		n	x ± SEM	x ± SEM	
	T	7	246.5 ± 8.0 mN	435.7 ± 24.3 mN	+76.8
NORMAL	$+\dot{T}$	7	39.9 ± 1.9 Ns^{-1}	66.7 ± 3.2 Ns^{-1}	+67.2
	$-\dot{T}$	7	18.3 ± 0.9 Ns^{-1}	34.6 ± 1.8 Ns^{-1}	+89.1
	T	7	237.7 ± 12.3 mN	150.1 ± 9.4 mN	-36.8
TREATED	$+\dot{T}$	7	39.3 ± 2.3 Ns^{-1}	24.3 ± 1.6 Ns^{-1}	-38.2
	$-\dot{T}$	7	16.5 ± 1.1 Ns^{-1}	7.2 ± 0.6 Ns^{-1}	-56.4

T, $+\dot{T}$, and $-\dot{T}$ were measured from the recorded traces. As in Part I, the contraction characteristics for a single twitch remain unchanged by creatine depletion however the values for the derivative of tension are higher in animals from Part II (see Table 5).

Twitch tension and $+\dot{T}$ are both nearly doubled in normal muscles and decreased by about 40% in depleted muscles following a 1 second tetanus. $-\dot{T}$ is also nearly doubled in normal muscles while in depleted muscles it is decreased by about 60%. This information is presented graphically in Figure 9.

For simplicity and ease of comparison the remainder of the data on twitch responses are expressed as a ratio of after to before the tetanus. Potentiation of the response is therefore indicated by a value greater than 1; depression of the response by a value less than 1.

2) Variable interval between the tetanus and subsequent twitch

Figure 10 shows the time course of posttetanic twitch potentiation and depression. The ratio of post- to pre-tetanic tension (T_2/T_1) again shows a long lasting potentiation in normal muscles and a short lasting depression in depleted muscles (Figure 10A). Depleted muscle recovers rapidly from the depression but rather than remain at pre-conditioning levels as observed with tetanic contractions in Figure 8, it develops

Figure 9. Recordings of twitch tension and its derivative before (left) and 200 msec after (right) a 1 second conditioning tetanus in normal (top) and creatine depleted (bottom) rat EDL muscles. In each case the lower trace is tension in newtons (N) and the upper trace is the derivative of tension in newtons per second ($Nsec^{-1}$).

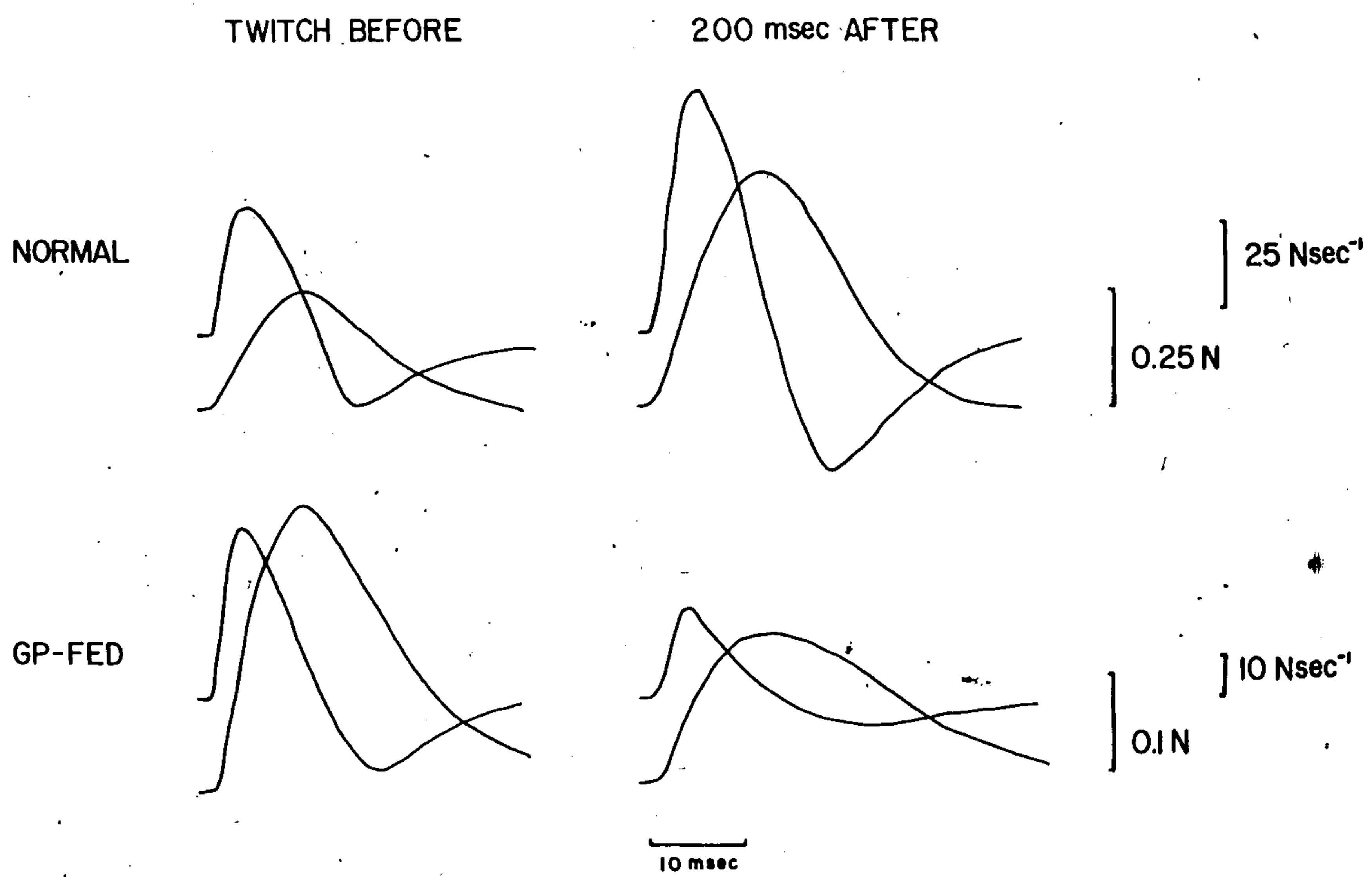
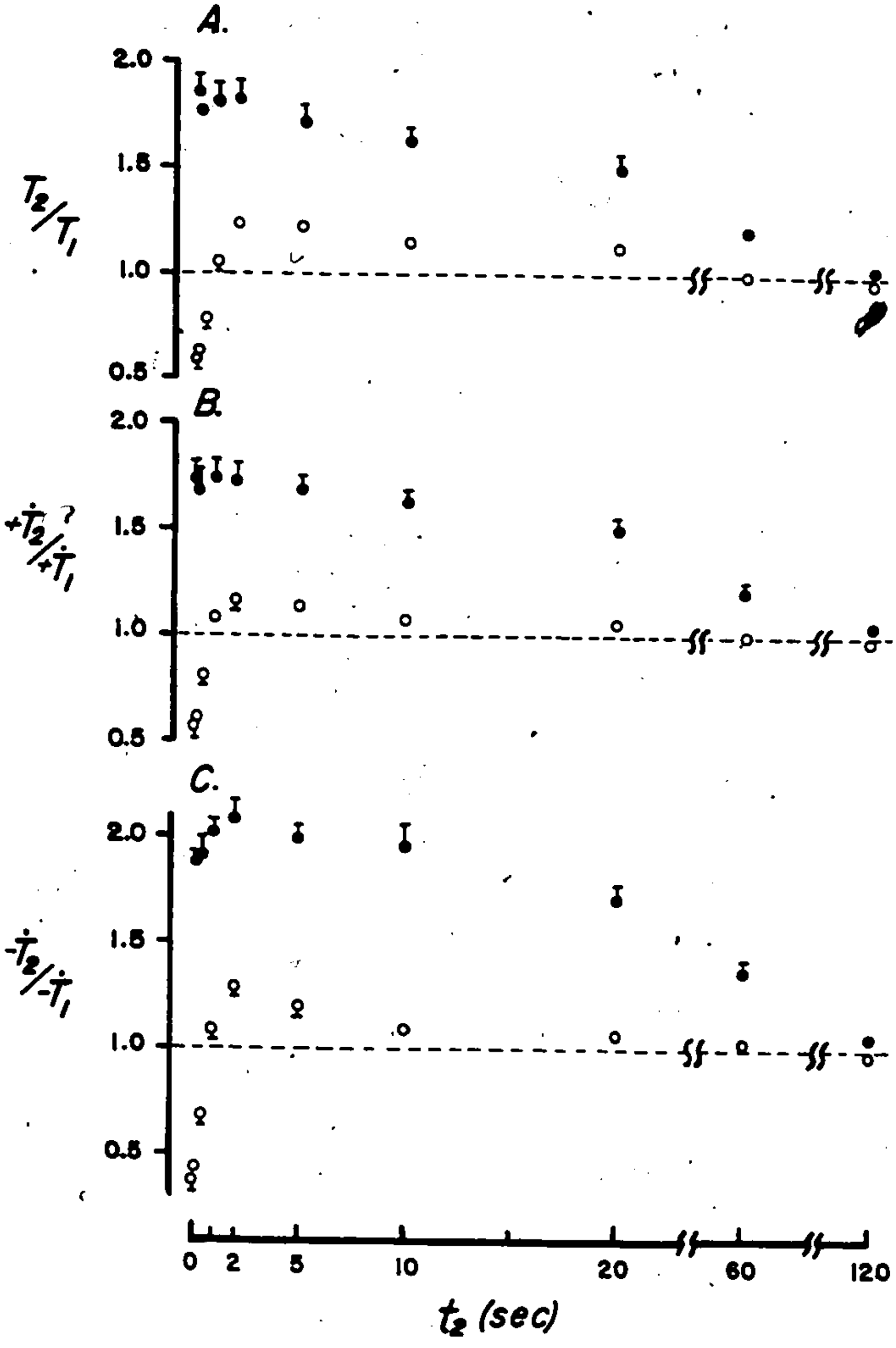

TWITCH BEFORE
200 msec AFTER
NORMAL
GP-FED
25 Nsec⁻¹
0.25 N
10 Nsec⁻¹
0.1 N
10 msec

Figure 10. Time course of the effects of a 1 second conditioning tetanus on the ratio of the A. twitch tension (T_2/T_1), B. maximum rate of tension development $(+\dot{T}_2/+\dot{T}_1)$, and C. the maximum rate of relaxation $(-\dot{T}_2/-\dot{T}_1)$ of twitches delivered 100 msec before and a variable interval after the tetanus in normal (filled circles; n=6) and creatine depleted (open circles; n=7) EDL muscles. Posttetanic stimuli were delivered at increasing intervals (t_2) from 100 msec to 120 seconds. When the 100 msec interval was repeated at the end of the protocol the response was very similar to that obtained at the start.


A.
2.0
1.5
1.0
0.5
T_2/T_1
B.
2.0
1.5
1.0
0.5
$+\dot{T}_2/+\dot{T}_1$
C.
2.0
1.5
1.0
0.5
$-\dot{T}_2/-\dot{T}_1$
0
2
5
10
20
60
120
t_2 (sec)

potentiation which, although of considerably less magnitude than normal muscles, is also long lasting.

The time course of the effects of the conditioning stimulus on twitch $+\dot{T}$ and $-\dot{T}$ is quantitatively similar to that just described for twitch tension and is shown in Figures 10B and 10C, respectively. The cross-over point from depression to potentiation in depleted muscles occurs consistently between the 0.5 and 1.0 second interval for all three twitch parameters. There appears to be at least two distinct phases in the time course of the responses in depleted muscles: an early rapid phase and a later slow phase.

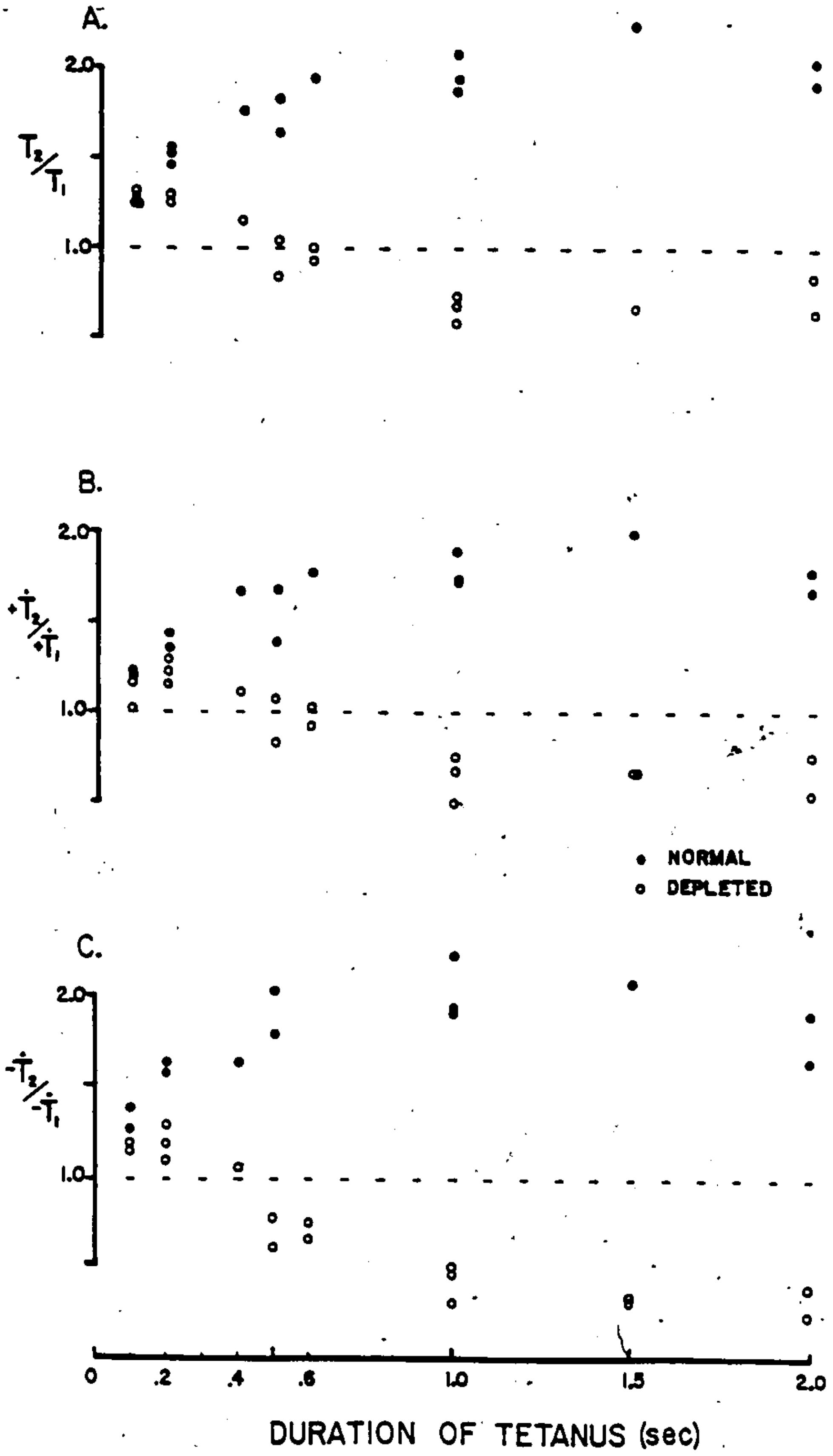
3) Variable duration of conditioning tetanus

The observation that early posttetanic depression of twitch T, $+\dot{T}$ and $-\dot{T}$ is reversible in creatine depleted EDL muscle led to the question of what effect the duration of the conditioning tetanus has on this depression and its reversal. With short tetanic durations, from 100 to 500 msec, the depleted muscles exhibit a small posttetanic twitch potentiation (about 20%) while longer durations exceeding 600 msec are accompanied by depression of twitch T, $+\dot{T}$, and $-\dot{T}$ (Figure 11).

4) Multiple pulses before and after the tetanus

In order to better distinguish the mechanisms behind PTP

Figure 11. Effect of varying the conditioning tetanus duration on the ratio of A. twitch tension (T_2/T_1), B. the maximum rate of tension development ($+\dot{T}_2/+\dot{T}_1$), and C. the maximum rate of relaxation ($-\dot{T}_2/-\dot{T}_1$) in normal (filled circles) and creatine depleted (open circles) EDL muscles. Duration of the tetanus is plotted on the abcissa and ranges from 0.1 to 2.0 seconds. Twitches were delivered 100 msec before and 200 msec after the tetanus. Each point is from one animal.


A.
2.0
T_2/T_1
1.0
B.
2.0
$+\dot{T}_2/+\dot{T}_1$
1.0
● NORMAL
○ DEPLETED
C.
2.0
$-\dot{T}_2/-\dot{T}_1$
1.0
0
.2
.4
.6
1.0
1.5
2.0
DURATION OF TETANUS (sec)

in normal muscles and PTD in depleted muscles, we looked at the posttetanic multiple pulse response. A preliminary series of experiments established that the maximum level of activation is achieved with four pulses 2.0 msec apart. That is, $+\dot{T}$ showed no further change with either an increase in pulse number or a change in pulse interval. The curves displayed in Figure 12 illustrate the rate of tension development for a single pulse compared with multiple pulses following the conditioning stimulus. Both normal and depleted muscles show an increased ability develop tension with increased activation irrespective of the energy demands imposed by the previous tetanus.

Table 7 contains the quantitative information on the multiple pulse responses. In normal muscles, T and $+\dot{T}$ are nearly doubled for a single twitch as shown previously in Table 6. The potentiating effect of the conditioning stimulus progressively declines as the number of pulses increases and is barely detectable in the four pulse response. In depleted muscles the depressing effect of the conditioning stimulus also progressively declines with increasing pulse number. T and $+\dot{T}$ of the single pulse response show about a 40% depression while $-\dot{T}$ is reduced by about 60%, again as shown previously in Table 6. In contrast, T is reduced by 17%, $+\dot{T}$ by 26%, and $-\dot{T}$ by 42% with the four pulse response. The curves in Figure 13 clearly show the decline of both potentiation and depression as the curves from normal and depleted muscles approach each other.

Figure 12. Recordings of the derivative of tension during 1, 2, 3, and 4 pulse contractions in normal (top) and creatine depleted (bottom) muscle before (left) and 200 msec after (right) a 1 second conditioning tetanus. Multiple pulses were delivered 2.0 msec apart.

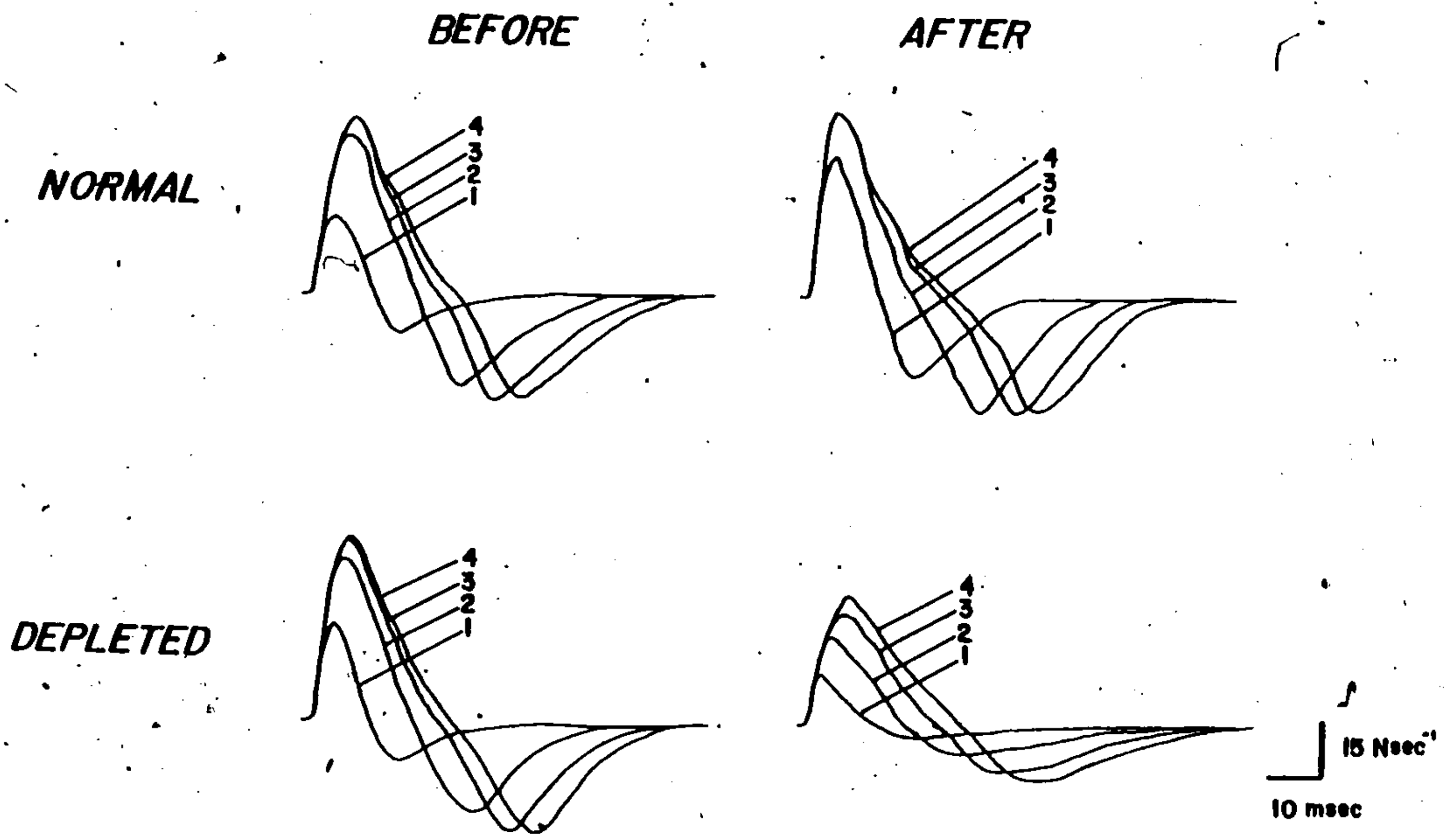

BEFORE
AFTER
NORMAL
DEPLETED
4
3
2
1
15 Nsec⁻¹
10 msec

Table 7. Multiple pulse response before and after a 1 second conditioning tetanus in normal and creatine depleted EDL muscles (n = 7 in all cases).

		NORMAL		DEPLETED	
N*		BEFORE	AFTER	BEFORE	AFTER
1	T	246.5± 8.0	435.7±24.3	237.7±12.3	150.1± 9.4
	$+\dot{T}$	39.9± 1.9	66.7± 3.2	39.3± 2.3	24.3± 1.6
	$-\dot{T}$	18.3± 1.0	34.6± 1.8	16.5± 1.1	7.2± 0.6
2	T	838.6±23.2	931.0±29.1	724.1±37.4	463.9±25.4
	$+\dot{T}$	85.0± 2.7	100.6± 1.9	76.4± 3.2	47.7± 2.1
	$-\dot{T}$	48.6± 2.4	59.2± 2.8	40.9± 2.6	17.7± 1.4
3	T	1083.8±21.8	1089.6±30.9	961.4±41.4	723.8±36.5
	$+\dot{T}$	96.4± 1.7	102.0± 2.2	88.2± 3.2	60.1± 2.4
	$-\dot{T}$	57.1± 3.2	65.1± 3.9	51.3± 3.0	26.3± 2.0
4	T	1129.5±26.1	1147.9±28.4	1055.8±41.2	871.6±39.6
	$+\dot{T}$	92.7± 1.5	99.9± 1.1	90.1± 2.9	65.8± 2.3
	$-\dot{T}$	58.2± 1.6	63.1± 2.9	53.3± 2.6	30.7± 2.2

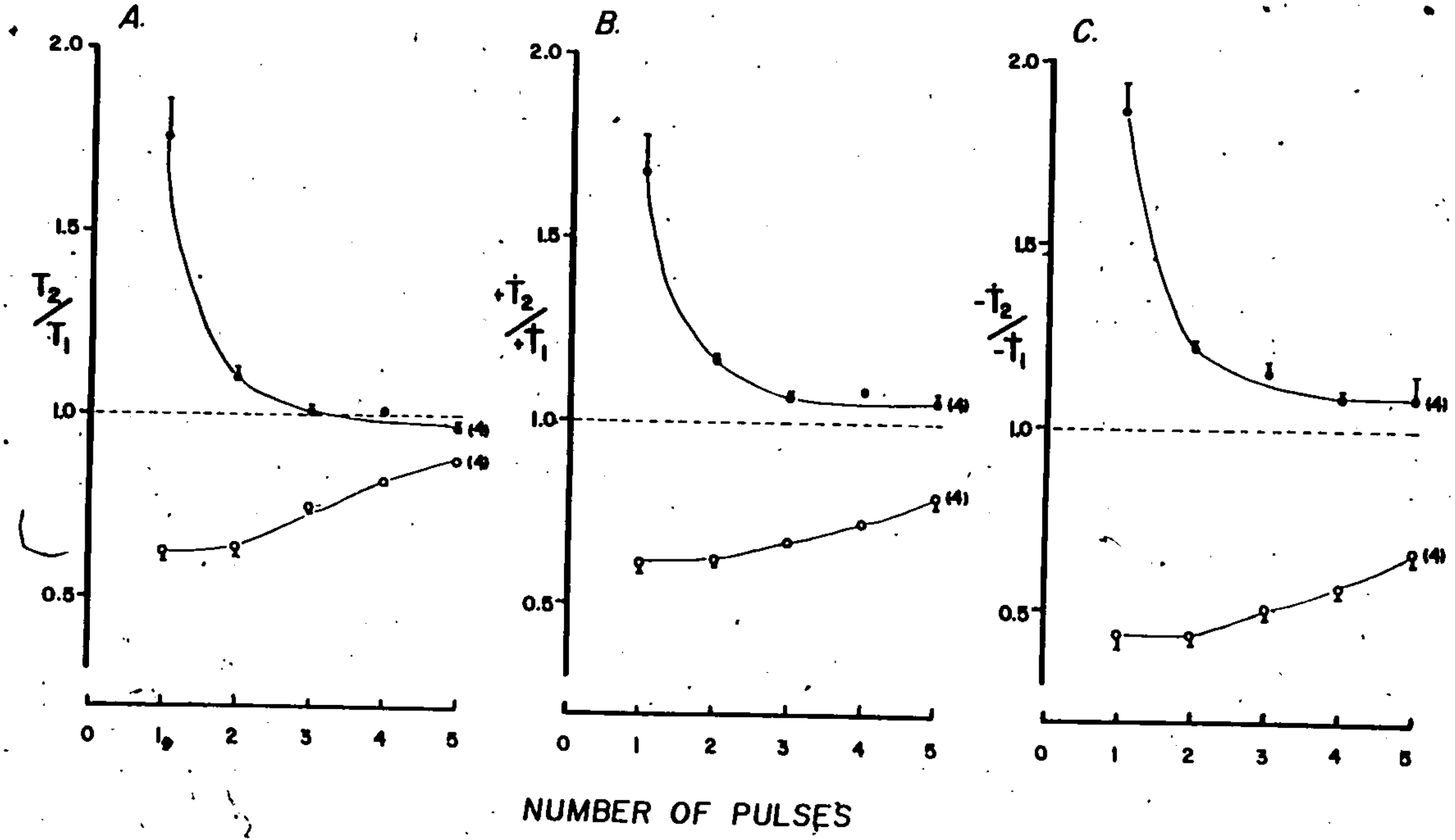
* N = the number of pulses

all values expressed as mean ± standard error of the mean

units for T are millinewtons (mN)

units for $\pm\dot{T}$ are newtons per second (Ns^{-1})

Figure 13. Effect of a 1 second conditioning tetanus on the ratio of A. tension (T_2/T_1), B. the maximum rate of tension development ($+\dot{T}_2/+\dot{T}_1$), and C. the maximum rate of relaxation ($-\dot{T}_2/-\dot{T}_1$) of muliple pulse responses delivered 100 msec before and 200 msec after the tetanus in normal (filled circles; n=6) and creatine depleted (open circles; n=7) EDL muscles. The number of pulses is plotted along the abcissa. Multiple pulses were delivered 2.0 msec apart.


A.
B.
C.
T2/T1
+T2/+T1
-T2/-T1
2.0
1.5
1.0
0.5
0
1
2
3
4
5
(4)
NUMBER OF PULSES

DISCUSSION

DEPLETION TECHNIQUE

1) Growth rate and muscle size

As outlined briefly in the introduction, GP feeding has been shown to have effects other than the alteration of creatine metabolism. Shields and Whitehair (1973) reported a decreased food intake, weight gain, and litter size in pregnant rats fed 1 or 2% GP in their diet during the last week of pregnancy. This effect on food intake and growth rate was not observed in male weanling rats fed 1% GP for 20 days.

In a subsequent investigation, Shields *et al.* (1975) observed that GP feeding results in decreased type II fibre size. Expressed as a ratio of mean fibre area (in square cm x 2.5×10^{-8}) to body weight, type II fibre size of gastrocnemius muscles from GP-fed rats was 2.27 ± 0.11 compared with 3.96 ± 0.27 in normal rats. Fitch *et al.* (1978) reported that plantaris muscles of GP-fed rats weighed only 71% of normal after adjusting for differences in body weight but that soleus muscles were of normal size. These findings indicate that both body weight and muscle size are decreased in response to GP feeding.

Since in the studies mentioned above all animals were given food *ad libitum*, it is difficult to determine whether GP itself has a specific effect on total body growth, whether the size effect is caused by decreased food intake, or both. In this present investigation, the same amount of chow was given to both control and treated rats each day to eliminate the effect of differences in caloric intake. This feeding technique has been described previously (Mainwood *et al.*, 1982a).

In spite of controlling food intake, we still observed a 12% reduction in the mean body weight of rats fed GP from 5 days old while those fed GP from weaning only were not significantly different from normal (Table 2). EDL muscle weight was reduced by 24% in the group fed GP from 5 days of age and by 9% in the group fed GP from weaning. Thus, although GP feeding from 5 days of age produces no further reduction in total muscle creatine (Table 1), it does seem to reduce total body growth and to more markedly reduce muscle size. It must be kept in mind however, that there are differences in duration of GP feeding between the two analogue-fed groups, and the above observations may involve a time-related effect.

A minor flaw in our controlled feeding technique is that during the first two days on solid food the GP-fed animals usually eat a few grams less than controls. A simple improvement may be to start off with a smaller quantity of chow and gradually increase it to 10 grams.

The fact that GP-feeding from weaning has no effect on body weight but significantly reduces muscle size suggests that GP does interfere with some aspect of muscle growth. It has been reported that creatine stimulates actin and myosin synthesis in differentiating cultured skeletal muscle cells (Ingwall *et al.*, 1972, 1974, 1976) however this work was later challenged and evidence was presented that creatine has no effect on contractile protein synthesis in developing muscle (Fry and Morales, 1980). It is still possible that a certain level of creatine is necessary to sustain normal muscle growth however further work must be done to determine if the observed growth retardation is due to reduction of muscle creatine, to a toxic effect of accumulated GP, or both. The possibility that GP accumulation exerts an effect on the molecular structure of muscle cells should be considered.

2) Biochemical changes

Our biochemical data clearly show that we successfully depleted the rat EDL muscle of creatine and creatine phosphate, however there are some differences between the assay results in Part I (Table 3) and Part II (Table 4). The largest discrepancy is in the total creatine content. Following hydrolysis of creatine phosphate, normal muscles contain 23.0 ± 1.4 μmol of total creatine per gram wet muscle weight in Part I and 30.6 ± 0.4 μmol·g^{-1} in Part II. This corresponds to about 9.1 and 18.9 μmol of free creatine per gram in Part I and II, respectively. A similar difference is seen in the mean total GP content betweenn the two treated groups: 13.4 ± 1.3 μmolg^{-1} in Part I and 29.5 ± 1.1 μmolg^{-1} in Part II. The values obtained for total creatine and GP in Part II are reasonably close to those values reported for the fast twitch plantaris muscle by Fitch *et al.* (1978): the total creatine content was about 32 μmolg^{-1} and the total GP content about 34 μmolg^{-1}. Shields and Whitehair (1973) measured about 37.1 μmolg^{-1} total creatine and about 33.1 μmolg^{-1} total GP in rat gastrocnemius.

Such a large difference in the mean creatine content between the two groups of control muscles and in the mean total GP content between the two groups of depleted muscles is difficult to account for and cannot be explained at this time.

An interesting result of GP feeding is that ATP levels are reduced (Fitch *et al.*, 1974, 1975; Mainwood *et al.*, 1982b). Our depleted EDL muscles had ATP levels ranging between 24% and 36% of normal. The reason for this reduction that apparently accompanies GP accumulation is unclear. However, ADP content is also reduced, by 25%, (Fitch *et al.*, 1974) which suggests a net loss of adenine. The possibility that GP interferes with adenine transport and/or metabolism remains an interesting point to pursue.

Muscles from GP-fed animals clearly have a reduced utilizable high energy phosphate pool (ATP + CrP). Although GPP can accumulate to levels in excess of 30 μmolg^{-1} (Fitch *et al.*, 1974, 1975; Petrofsky and Fitch, 1980), it is generally accepted that this phosphorylated analogue is a poor substrate for creatine kinase and is insignificant as a high energy phosphate donor during muscular activity (Fitch and Chevli, 1975; Chevli and Fitch, 1979; Mainwood *et al.*, 1982a).

Perhaps more important than the reduction in high energy phosphates is the effect the altered relative energy substrate concentrations (ATP, ADP, Cr, and CrP) have on the free energy change of ATP hydrolysis ($-dG/d\xi$). As discussed by Curtin *et al.* (1974), $dG/d\xi$ is a measure of the chemical energy available to do work. The estimated $-dG/d\xi$ is reduced by 10.2% in Part I and by 7.6% in Part II. According to Dawson *et al.* (1980) very small changes in $-dG/d\xi$ can have large effects on those processes requiring ATP. This becomes important when considering the possible mechanisms behind the observed contractile changes and will be discussed at greater length in the appropriate section of the discussion.

3) Basic contractile parameters

As shown in Table 5 (Part I) and Table 6 (Part II), basic twitch parameters of normal EDL muscles are not different from those of depleted muscles. Since the depleted muscles are smaller, yet they develop the same absolute tension as normal muscles, the tension development per unit area must then be greater than normal. Although the mean normalized values for tension and its derivative are higher than normal, the variability is large and the difference is not statistically significant.

The tetanic contraction characteristics on the other hand, show a decreased ability of depleted muscles to develop tension as well as a slowing of both the rate of tension development and the rate of relaxation (Table 5). The rate of relaxation is the parameter most affected by GP feeding and is the only one to remain significantly different after normalizing the data for cross-sectional area.

The maximum rates of tension development and relaxation are greater in Part II than in Part I, giving the impression that muscles from Part II are faster. Although a detailed statistical comparison was not made, the times to peak tension do not appear different between Parts I and II. The most likely explanation seems to lie in the improved response characteristics of the Kulite strain gauge used in Part II.

POSTTETANIC EFFECTS ON THE SINGLE BRIEF TETANUS

In Part I of the contraction studies we showed that the fast twitch rat EDL exhibits similar contractile characteristics to the diaphragm when depleted of creatine: namely, following a single tetanus the maximum rate of relaxation ($-\dot{T}$) is slower than normal and in response to a series of tetanic stimuli (3 per second) the maximum rate of tension development ($+\dot{T}$) decreases as the contraction number increases. In normal muscles $+\dot{T}$ increases with the contraction number (Figure 5). Further information obtained from this muscle preparation is that the maximum effect on $+\dot{T}$ is reached after a total stimulation time of 1 second and is independent of series or continuous tetanic stimulation (Figure 6).

The relatively long lasting potentiation of $+\dot{T}$ in normal muscle and the rapidly reversed depression of $+\dot{T}$ in depleted muscle during the test tetanus (Figure 8) indicate that different mechanisms underlie the effects of a conditioning stimulus in the two muscle groups.

POSTTETANIC TWITCH RESPONSE

In Part II of the contraction studies we examined the effect of a tetanus on a subsequent twitch contraction in normal and depleted muscles. The striking effect of tetanic stimulation on twitch responses has been known for some time and we became interested in this as a potentially more sensitive indicator of the effects of decreased creatine phosphate levels on the contraction process.

Following a 1 second tetanus at 100 Hertz, all three twitch parameters (T, $-\dot{T}$, and $+\dot{T}$) are nearly doubled in normal muscles while in creatine depleted muscles T and $+\dot{T}$ are decreased by 40% and $-\dot{T}$ by 60% (Figure 10). Once again, the rate of relaxation seems to be the contraction parameter most affected by creatine depletion. Posttetanic twitch potentiation (PTP) has been previously characterized in the fast twitch rat EDL (Close and Hoh, 1969; Krarup, 1981) and posttetanic twitch depression (PTD) has been described in slow twitch muscles of the rat (Close and Hoh, 1969) and cat (Buller *et al.*, 1981). Reversal of PTP in fast twitch muscles does not appear to have been reported previously in the literature.

Close and Hoh (1968) observed no change in contraction time of the potentiated twitch delivered 10 seconds after a 1 second 300 Hertz tetanus (twitch tension was about doubled). Although a detailed analysis of this parameter was not made in this investigation, the fact that the same potentiation is observed in twitch T and +T indicates that the twitch contraction time remains unchanged after the tetanus. Krarup (1981) found little change in twitch contraction time 2 seconds after a 1.5 second 125 Hertz tetanus and twitch tension was more than doubled. Thus the normal EDL muscle develops more twitch tension at a faster rate and also relaxes faster following 1 second of tetanic stimulation. By comparison, depleted muscle develops about half the pretetanic tension at about half the rate and relaxation is substantially prolonged. The posttetanic depression observed in slow twitch muscle is quite different from this. In the cat soleus, a marked depression in twitch tension development occurs, but is accompanied by shortened contraction and half relaxation times and only a slight depression in the maximum rate of tension development occurs following a 1 second 100 Hertz tetanus (Buller *et al.*, 1981). This difference, plus the fact that the depleted muscles show no characteristics of slowing during a single twitch (Table 6), indicates that creatine depletion is probably not associated with fibre type conversion, *i.e.*, from fast to slow.

The difference in the time course of recovery from the posttetanic effects between normal and depleted muscle is quite striking (Figure 10). PTP in normal muscles declines slowly and has disappeared by about 2 minutes while PTD in depleted muscles recovers within 1 second and is followed by a PTP about one-third normal which also disappears by about 2 minutes. Close and Hoh (1968) observed complete recovery from PTP 4 minutes after a 1 second 300 Hertz tetanus and Krarup (1981) reported a recovery from a 1.5 second 167 Hertz tetanus was complete within 5-10 minutes in normal rat EDL. Both Close and Hoh (1968) and Krarup (1981) elicited a series of twitch contractions following a single conditioning tetanus to obtain their PTP time course data which likely delays recovery from potentiation and obscures somewhat the real effect the time interval has on the twitch response. It is evident however, that PTP in normal muscles consistently recovers within minutes and that depleted EDL muscles exhibit two distinct phases to the posttetanic recovery process: early depression and later potentiation.

Interestingly, this early depression is replaced by a small potentiation when tetanic duration is less than 500 msec (Figure 11). At very short tetanic durations (100 msec) normal and depleted muscles develop the same degree of potentiation but their responses begin to diverge at durations of 200 msec. Buller *et al.* (1981) reports that PTD is maximal in the cat soleus following a 300 msec 100 Hertz tetanus, it requires more than 100 seconds for complete recovery, and the recovery shows both an early fast and a later slow phase. Although there are many differences in the PTD between the creatine depleted EDL and the normal soleus, there is one important similarity. Both exhibit an early fast phase of recovery with a time course of about 1 second.

POSSIBLE MECHANISMS BEHIND PTP AND PTD

The posttetanic twitch response in creatine depleted muscle seems to involve an interplay between two opposing processes which are dependent on tetanus duration. With very short tetanic durations potentiation dominates and as the duration increases, depression becomes dominant. The early twitch depression following long tetanic durations suggests that either the energy supply becomes exhausted and limits the rate of cross bridge turnover the muscle can support or that a prolonged stimulus somehow limits the activation step in a subsequent response. In this paper activation is defined as the process that *switches on* or *activates* the myofibrils and represents the steps between the muscle action potential and the development of tension. Whatever the mechanism is that causes PTD, clearly it is rapidly reversible.

To distinguish between these two possible mechanisms of PTD we investigated whether we could increase the posttetanic response in depleted muscle by increasing the number of pulses. A variable number of pulses (from 1 to 4) were delivered to elicit a well fused summated contraction before and after a 1 second tetanus. Four pulses 2 msec apart were required in both normal and depleted muscles for complete activation. If the energy limiting hypothesis is true, we reasoned that the PTD observed in depleted muscles would become even greater if a greater energy demand is imposed upon the muscle following the tetanus. Table 7 shows the posttetanic tension was increased more than 5 times in depleted muscles by increasing activation with 4 pulses. This is not just a result of a longer contraction since the maximum rate of tension development ($+\dot{T}$) is increased more than 2.5 times. By comparison, tension is increased only 2.5-fold and $+\dot{T}$ only 1.5-fold in normal muscle. Thus, normal muscle is closer to being maximally activated by a single posttetanic stimulus than depleted muscle. The fact that depression in depleted muscle almost disappears when activation is maximal with 4 pulses (Figure 12) makes it quite clear that the level of ATP in the myofibrils is not the most important factor in the early PTD and that the myofibrils are not adequately activated by a single stimulus immediately after a long tetanus.

This decreased activation in depleted muscle implies that either some component of excitation-contraction coupling (*e.g.*, calcium release) is reduced or the sensitivity of the myofibrils to calcium is altered, or both.

The above arguments address the changes observed immediately following a 1 second tetanus (*i.e.*, within the first 500 msec). The later changes, after 1 to 2 seconds, are long lasting in comparison and are similar to but smaller than those observed in normal muscle. The most likely explanation for these observations is that some type of structural or molecular change occurs in both normal and depleted muscles. Phosphorylation of myosin light chain is a likely candidate for such a molecular change and could occur in both muscles but to different extents. Manning and Stull (1979, 1982) examined the phosphorylation of light chain myosin in isolated rat EDL in relation to tetanic stimulation and found that following a 1 second tetanus, the maximal increase in phosphorylation corresponds temporally to the maximal potentiated twitch response and both decline at similar rates. A temporal relationship was also reported between myosin light chain phosphorylation (MLCP) and the potentiated twitch response during low frequency stimulation at 5 Hertz in *in situ* rat gastrocnemius muscle (Klug *et al.*, 1982). These observations suggest an interesting role for the phosphorylated light chain in modifying contraction during potentiation. Since the aequorin response during the posttetanic potentiated twitch in frog muscle indicates that the amount of calcium released by a single action potential is decreased by a previous tetanic stimulus (Blinks *et al.*, 1978; Figure 8) MLCP may modulate contraction by increasing myofibrillar sensitivity to calcium. The mechanism by which MLCP could lead to such a change in calcium sensitivity cannot be established at this time.

If potentiation can and does occur in the depleted muscles, why then is it delayed and what is responsible for the early depression? Any potential answers to these questions are purely speculative at this point since there is no direct evidence to support them.

The delayed potentiation could be explained by two things: either it is superimposed upon a much greater depression which temporarily masks it, or the process causing potentiation (presumably MLCP) is delayed. Because early potentiation was observed following the short tetani, the first possibility is most likely.

The early depression, as mentioned earlier, is evidently due to a process that is rapidly reversible. It is generally accepted that while ATP hydrolysis generates H+ ions (Alberty, 1969), CrP hydrolysis consumes them (Curtin and Woledge, 1978). In normal muscles, while ATP is being recycled during activity, a H+ ion gradient may be generated by creatine phosphate breakdown making the microenvironment around the myofibrils relatively alkaline. Evidence for alkalinity in muscle fibres following activity was presented several decades ago (Meyerhof and Lohmann, 1928; Fiske and Subbarow, 1929a; Dubuisson, 1954). Depleted muscles, with their CrP levels reduced to 4% of normal, have a reduced ability to buffer the H+ ions generated upon ATP hydrolysis and a sudden burst of activity could generate a transient local pH gradient in the opposite direction. The effect of H+ ions on force development in rabbit (Donaldson and Hermansen, 1978) and frog (Robertson and Kerrick, 1979) muscles has been investigated. Decreasing the pH apparently shifts the force-pCa curve toward increasing calcium concentrations. Thus, as the [H+] increases, more calcium is required to achieve the same tension development. Such a mechanism could account for the time course of PTD since a local proton accumulation could rapidly disappear by diffusion.

Table 7 shows once again that the parameter most sensitive to tetanic stimulation is the rate of relaxation. $-\dot{T}$ is reduced by 56% in depleted muscles and increased by 89% in normal muscles during a single posttetanic twitch. Relaxation can be slowed by reducing the rate of cross bridge detachment, by reducing the off rate of calcium from troponin C, and by reducing the rate of calcium reuptake into the sarcoplasmic reticulum. Since the ATP availability is sufficient to support a greater posttetanic contraction with multiple than with single stimuli, it is unlikely that ATP levels around the myofibrils become low enough to limit cross bridge kinetics during either tension development or relaxation.

Therefore, our evidence most strongly suggests that the two components of contraction most affected by creatine depletion are: 1. calcium release → myofibril activation and 2. myofibril deactivation → calcium reuptake which alter tension development and relaxation, respectively.

Since the evidence and the arguments presented above tend to negate the hypothesis that the reduced energy stores limit cross bridge cycling, these reduced stores must be affecting contractile performance in some other way. A simple calculation of the expected energy cost of a 1 second tetanus based on a contraction maintenance heat of 132 mJg^{-1}s^{-1} (Wendt and Gibbs, 1973) and an enthalpy change of 34 kJmol^{-1} for CrP (Curtin and Woledge, 1978), shows that about 4 μmol ~P/gram of muscle is required. The ATP and CrP supply would be exhausted before the 1 second tetanus is over at this energy utilization rate. What is happening in the depleted muscle during the tetanus that allows the maintenance of tension and prevents rigor? Do these muscles undergo some metabolic adaptive process whereby ATP utilization is altered? Is there a rapid regeneration source for ATP that can more quickly switch on the metabolic enzymes? Are the levels of the metabolic enzymes increased? Without knowing the answers to these questions it is difficult to evaluate the relative importance of the ATP and CrP store as an energy buffer in the depleted muscles.

Another potentially important consideration is that some fibre types may be more sensitive to creatine depletion than others. It was mentioned earlier in this thesis that the rat EDL has a mixed fibre composition (59% FOG, 38% FG, 3% SO) and it is possible that the posttetanic effects we observe in analogue-fed rats result from a dysfunction in one of these populations of fibres.

Regardless of how these muscles may adapt to chronic CrP and ATP deficiency, it is likely that during the 1 second tetanus the ATP level decreases and the ADP level increases. As mentioned previously, changes in the ATP/ADP ratio will affect

$-dG/d\xi$ of ATP hydrolysis which represents the effective driving force for contraction (*i.e.*, the energy available to do work). ATP hydrolysis occurs at many points throughout the cell and drives processes both directly and indirectly involved in the mechanics of muscle contraction. Since $-dG/d\xi$ is up to 10% less in resting depleted muscles (Table 4) it may become limiting during the potentiation process and account for the smaller PTP.

It has been estimated that muscle spends about 1 mcal/gram for calcium transport in a single twitch (Kobayashi and Sugi, 1980) and total energy output is 2-3 mcal/gram (Hasselbach and Oetliker, 1983). With one-third of the total cellular energy needed for calcium transport, the change in ATP/ADP in depleted muscle should dramatically affect calcium cycling. This leads to a possible explanation of the effect creatine depletion has on activation and deactivation of contraction. Hasselbach and Oetliker (1983) present the following equation to describe the relationship between ATP and the calcium pump cycle:

$$2Ca_o + ATP \rightleftharpoons 2Ca_i + ADP + P_i$$

where Ca_o is the free myoplasmic calcium and Ca_i is the stored releasable calcium concentration inside the sarcoplasmic reticulum. Basic chemistry tells us that,

$$K_{eq} = \frac{[Ca_i]^2[ADP][P_i]}{[Ca_o]^2[ATP]} .$$

This means that the releasable fraction of calcium within the sarcoplasmic reticulum (Ca_1) will decline during the 1 second tetanus if the ADP levels are allowed to rise assuming this system remains close to its equilibrium. The successive reactivations during the 1 second tetanus in depleted muscle result in a progressive decline in the ATP/ADP ratio which limits calcium reuptake. Progressively more calcium is left in the myoplasm to be mopped up by calcium binding proteins, less is replaced inside the sarcoplasmic reticulum, resulting in a progressive decline in the amount of calcium released following an action potential. Increased myoplasmic calcium levels will slow the rate of calcium dissociation from troponin C since more calcium binding sites are occupied. At the end of the tetanus then, the rate of relaxation is considerably slowed and during a subsequent twitch the rate of tension development is limited by inadequate calcium release. A transient proton load generated by ATP hydrolysis could exacerbate this effect on tension development. These two processes could conceivably overwhelm the potentiation mechanism and cause the observed transient PTD.

It has been mentioned several times in this discussion that the maximum rate of relaxation seems to be the most sensitive contractile parameter to the effects of creatine depletion. It is not clear why the deactivation step should be affected more than the activation step if altered calcium dynamics is the mechanism responsible. However, it was recently reported that ADP may exert an inhibitory influence on cross bridge detachment

(Dantzig *et al.*, 1984) which could add to an already slowed calcium reuptake.

CONCLUDING REMARKS

We are proposing that, following a tetanus, creatine depletion primarily affects the activation and deactivation steps of contraction in fast twitch muscles and that this effect may be expressed through altered calcium dynamics resulting from an alteration in the relative energy substrate concentrations. Other potentially important effects are a depressed free energy change of ATP hydrolysis, and an inability to buffer the H^+ ions generated upon ATP hydrolysis. It seems that the primary role of the creatine-creatine phosphate system in fast twitch muscles is, in fact, to buffer changes in the ATP/ADP ratio.

REFERENCES

1. Alberty, R.A. (1969). Standard Gibbs free energy, enthalpy, and entropy changes as a function of pH and pMg for several reactions involving adenosine phosphates. *J. Biol. Chem. 244*: 3290-3302.
2. Bessman, S.P. and Fonyo, A. (1966). The possible role of the mitochondrial-bound creatine kinase in regulation of mitochondrial respiration. *Biochim. Biophys. Res. Commun. 22*: 597-602.
3. Bessman, S.P. and Geiger, P.J. (1981). Transport of energy in muscle: The phosphorylcreatine shuttle. *Science 211*: 448-452.
4. Blinks, J.R., Rüdel, R., and Taylor, S.R. (1978). Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. *J. Physiol. 277*: 291-323.
5. Bloch, K. and Schoenheimer, R. (1941). The biological precursors of creatine. *J. Biol. Chem. 138*: 167-194.
6. Bonas, J.E., Cohen, B.D., and Natelson, S. (1963). Separation and estimation of certain guanidino compounds, application to human urine. *Microchem. J. 7*: 63-77.
7. Borsook, H. and Dubnoff, J.W. (1941). The formation of glycocyamine in animal tissues. *J. Biol. Chem. 138*: 389-403.
8. Buller, A.J., Kean, C.J.C., Ranatunga, K.W. and Smith, J.M. (1981). Post-tetanic depression of twitch tension in the cat soleus muscle. *Exp. Neurol. 73*: 78-89.

9. Cain, D.F. and Davies, R.E. (1962). Breakdown of ATP during a single contraction of working muscle. *Biochem. Biophys. Res. Commun.* 8: 361-366.

10. Cantoni, G.L. and Vignos, P.J. Jr. (1954). Enzymatic mechanism of creatine synthesis. *J. Biol. Chem.* 209: 647.

11. Chevli, R. and Fitch, C.D. (1979). β-GPA and β-GPAP as substrates for creatine kinase. *Biochem. Med.* 21: 162-167.

12. Chevreul, E.C. (1835). Untersuchungen uber die chemische Zusammensetzung der Fleischbrühe. *J. Prakt. Chem.* 6: 120.

13. Close, R. and Hoh, J.F.Y. (1968). The after effects of repetitive stimulation on the isometric twitch contraction of the rat fast skeletal muscle. *J. Physiol.* 197: 461-477.

14. Close, R. and Hoh, J.F.Y. (1969). Post-tetanic potentiation of twitch contractions of cross-innervated rat fast and slow muscles. *Nature* 221: 179-181.

15. Crow, M.T. and Kushmerick, M.J. (1983). Regulation of energetics and mechanics by myosin light chain phosphorylation in fast-twitch skeletal muscle. *Fed. Proc.* 42: 14-20.

16. Curtin, N.A., Gilbert, C., Kretzschmar, K.M., and Wilkie, D.R. (1974). The effect of the performance of work on total energy output and metabolism during muscular contraction. *J. Physiol.* 238: 455-472.

17. Curtin, N.A. and Woledge, R.C. (1978). Energy changes and muscular contraction. *Physiol. Rev.* 58: 690-761.

18. Dantzig, J.A., Hibbard, M.G., Goldman, Y.E. and Trentham, D.R. (1984). ADP slows cross-bridge detachment rate induced by photolysis of caged ATP in rabbit psoas muscle fibres. *Biophys. J. 45*: 8a. (abstract)

19. Dawson, M.J. Gadian, D.G. and Wilkie, D.R. (1980). Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance. *J. Physiol. 299*: 465-484.

20. Dhalla, N.S., Yates, J.C., Walz, D.A., McDonald, V.A. and Olson, R.E. (1972). Correlation between changes in the endogenous energy stores and myocardial function due to hypoxia in the isolated perfused rat heart. *Can. J. Physiol. Pharmacol. 50*: 333-345.

21. Donaldson, S.K.B. and Hermansen, L. (1978). Differential, direct effects of H^+ on Ca^{2+}-activated force of skinned fibres from the soleus, cardiac and adductor magnus muscles of rabbits. *Pflugers Arch. 376*: 55-65.

22. Dubuisson, M. (1954). *Muscular contraction.* A Monograph in: The Bannerstone Division of American Lectures in Biochemistry and Biophysics. Charles C. Thomas, Publisher. Springfield, Illinois.

23. Eggleton, P. and Eggleton, G.P. (1927). The inorganic phosphate and a labile form of organic phosphate in the gastrocnemius of the frog. *Biochem. J. 21*: 190.

24. Engelhardt, V.A. and Lyubimova, M.N. (1939). Myosin and adenosinetriphosphatase. *Nature 144*: 668.

25. Ennor, A.H. and Rosenberg, H. (1952). The determination and distribution of phosphocreatine in animal tissues. *Biochem. J. 51*: 606–610.

26. Fiske, C.H. and Subbarow, Y. (1927). The nature of the inorganic phosphate in voluntary muscle. *Science 65*: 401.

27. Fiske, C.H. and Subbarow, Y. (1929a). Phosphocreatine. *J. Biol. Chem. 81*: 629.

28. Fiske, C.H. and Subbarow, Y. (1929b). Phosphorus compounds of muscle and liver. *Science 70*: 381.

29. Fitch, C.D. and Shields, R.P. (1966). Creatine metabolism in skeletal muscle. I. Creatine movement across muscle membranes. *J. Biol. Chem. 241*: 3611–3614.

30. Fitch, C.D., Shields, R.P., Payne, W.F. and Dacus, J.M. (1968). Creatine metabolism in skeletal muscle. III. Specificity of the creatine entry process. *J. Biol. Chem. 243*: 2024–2027.

31. Fitch, C.D., Jellinek, M. and Mueller, E.J. (1974). Experimental depletion of creatine and phosphocreatine from skeletal muscle. *J. Biol. Chem. 249*: 1060–1063.

32. Fitch, C.D., Jellinek, M., Fitts, R.H., Baldwin, K.M., and Holloszy, J.O. (1975). Phosphorylated β-guanidinopropionate as a substitute for phosphocreatine in rat muscle. *Am. J. Physiol. 228*: 1123–1125.

33. Fitch, C.D. and Chevli, R. (1975). Measurement of β-guanidinopropionate and phosphorylated β-guanidinopropionate in tissues. *Anal. Biochem. 68*: 196–201.

34. Fitch, C.D., Chevli, R., Petrofsky, J.S. and Kopp, S.J. (1978). Sustained isometric contraction of skeletal muscle depleted of

phosphocreatine. *Life Sciences 23*: 1285-1292.

35. Fry, D.M. and Morales, M.F. (1980). A reexamination of the effects of creatine on muscle protein synthesis in tissue culture. *J. Cell Biol. 84*: 294-297.

36. Gudbjarnason, S., Mathes, P. and Ravens, K.G. (1970). Functional compartmentation of ATP and CrP in heart muscle. *J. Mol. Cell. Cardiol. 1*: 325-339.

37. Hasselbach, W. and Oetliker, H. (1983). Energetics and electrogenicity of the sarcoplasmic reticulum calcium pump. *Ann. Rev. Physiol. 45*: 325-339.

38. Hill, A.V. (1965). *Trails and Trials in Physiology*. Edward Arnold (Publishers) Ltd. London. pp.245.

39. Ingwall, J.S., Morales, M.F. and Stockdale, F.E. (1972). Creatine and the control of myosin synthesis in differentiating skeletal muscle. *Proc. Nat. Acad. Sci. 69*: 2250-2253.

40. Ingwall, J.S., Weiner, C.D., Morales, M.F., Davis, E. and Stockdale, F.E. (1974). Specificity of creatine in the control of muscle protein synthesis. *J. Cell Biol. 63*: 145-151.

41. Ingwall, J.S. (1976). Creatine and the control of muscle-specific protein synthesis in cardiac and skeletal muscle. *Circ. Res. 38(Suppl 1)*: I-115-123.

42. Jacobs, H., Heldt, H.W. and Klingenberg, M. (1964). High activity of creatine kinase in mitochondria from muscle and brain and evidence for a separate mitochondrial isozyme of creatine kinase. Biochem. Biophys. Res. Commun. 16: 516-521.

43. Jacobus, W.E. (1980). Myocardial energy transport. Current concepts of the problem. In: *Heart Creatine Kinase*. W.E.

Jacobus and J.S. Ingwall *eds.* Williams and Wilkins Co., Baltimore, Md. pp. 1-6.

44. Jacobus, W.E. and Lehninger, A.L. (1973). Creatine kinase of rat heart mitochondria. Coupling of creatine phosphorylation to electron transport. *J. Biol. Chem.* 248: 4803-4810.

45. Klug, G.A., Botterman, B.R. and Stull, J.T. (1982). The effect of low frequency stimulation on myosin light chain phosphorylation in skeletal muscle. *J. Biol. Chem.* 257: 4688-4690.

46. Kobayashi, T. and Sugi, H. (1980). Measurement of heat production during summation of isometric contraction in frog skeletal muscle with an infrared radiometer. *Jpn. J. Physiol.* 30: 617-629.

47. Krarup, C. (1981). Enhancement and diminuition of mechanical tension evoked by staircase and by tetanus in rat muscle. *J. Physiol.* *311*: 355-372.

48. Lamprecht, W. and Stein, P. (1965). Creatine phosphate. In: *Methods of Enzymatic Analysis.* ed. H.U. Bergmeyer. pp. 610-616. Academic Press, N.Y.

49. Lohmann, K. (1929). Uber die Pyrophosphatfraktion im Muskel. *Naturwissensch.* *17*: 624.

50. Lundsgaard, E. (1930). Untersuchungen über Muskelkontraktion ohne Milchsäure. *Biochem. Z.* *217*: 162.

51. Mainwood, G.W., Alward, M. and Eiselt, B. (1982a). The effects of metabolic inhibitors on the contraction of creatine depleted muscle. *Can. J. Physiol. Pharmacol.* *60*: 114-119.

52. Mainwood, G.W., Alward, M. and Eiselt, B. (1982b). Contractile characteristics of creatine depleted rat diaphragm. *Can. J.*

Physiol. Pharmacol. 60: 120-127.

53. Manning, D.R. and Stull, J.T. (1979). Myosin light chain phosphorylation and phosphorylase a activity in rat extensor digitorum longus muscle. *Biochem. Biophys. Res. Commun.* 90: 164-170.

54. Manning, D.R. and Stull, J.T. (1982). Myosin light chain phosphorylation-dephosphorylation in mammalian skeletal muscle. *Am. J. Physiol.* 242: C234-C241.

55. Meyerhof, O. and Lohmann, K. (1928). Uber die naturlichen Guanidinophosphorsäuren (Phosphagene) in der quergestreiften Muskulatur. II Die physikalisch-chemischen Eigenschaften der Guanidinophosphorsäure. *Biochem. Z.* 196: 49.

56. Petrofsky, J.S. (1979). Sequential motor unit stimulation through peripheral motor nerves. *Med. Biol. Eng. Comput.* 17: 87-93.

57. Petrofsky, J.S. and Fitch, C.D. (1980). Contractile characteristics of skeletal muscles depleted of phosphocreatine. *Pflugers Arch.* 384: 123-129.

58. Robertson, S.P. and Kerrick, W.G.L. (1979). The effects of pH on Ca^{2+}-activated force in frog skeletal muscle fibers. *Pflugers Arch.* 380: 41-45.

59. Rowley, G.L., Greenleaf, A.L. and Kenyon, G.L. (1971). On the specificity of creatine kinase. New Glycocyamines and Glycocyamine analogs related to creatine. *J. Am. Chem. Soc.* 93: 5542-5551.

60. Sakaguchi, S. (1925). Uber eine neue Farbenreaktion von Protein und Arginin. *J. Biochem.* 5: 25-31.

61. Saks, V.A., Lipina, V.N., Smirnov, V.N. and Chazov, E.I. (1976). Studies of Energy Transport in Heart Cells. The Functional Coupling between Mitochondrial Creatine Phosphokinase and ATP-ADP Translocase: Kinetic Evidence. *Arch. Biochem. Biophys. 173*: 34-41.

62. Saks, V.A., Rosenshtraukh, L.V., Smirnov, V.N. and Chazov, E.I. (1978). Role of creatine phosphokinase in cellular function and metabolism. *Can. J. Physiol. Pharmacol. 56*: 691-706.

63. Seraydarian, M.W., Abbott, B.C. and Williams, E.B. (1961). Studies on the frog sartorius at different stages of activity. *Biochim. Biophys. Acta 46*: 355-363.

64. Shields, R.F. and Whitehair, C.K. (1973). Muscle creatine: *In vivo* depletion by feeding β-guanidinopropionic acid. *Can. J. Biochem. 51*: 1046-1049.

65. Shields, R.P., Whitehair, C.K., Carrow, R.E., Heusner, W.W. and van Huss, W.D. (1975). Skeletal muscle function and structure after depletion of creatine. *Lab. Invest. 33*: 151-158.

66. Spande, J.I. and Schottelius, B.A. (1970). Chemical basis of fatigue in isolated mouse soleus muscle. *Am. J. Physiol. 219*: 1490-1495.

67. Swanson, P.D. (1967). The particulate adenosine triphosphate-creatine phosphotransferase from brain: its distribution in subcellular fractions and its properties. J. Neurochem. 14: 343-356.

68. Szent-Györgyi, A. (1945). Studies on muscle. *Acta Physiol. Scand.* 9(*Suppl.25*): 25.

69. Turner, D.C., Walliman, T. and Eppenberger, H.M. (1973). A protein that binds specifically to the M-line of skeletal muscle is identified as the muscle form of creatine kinase. *Proc. Nat. Acad. Sci. USA* 70: 702-705.

70. Walker, J.B. (1979). Creatine: Biosynthesis, regulation, and function. In: *Advances in Enzymology*. vol 50. Alton Meister ed. John Wiley & Sons. New York. pp. 177-242.

71. Wallimann, T., Turner, D.C. and Eppenberger, H.M. (1977). Localization of creatine kinase isoenzymes in myofibrils. I. Chicken skeletal muscle. *J. Cell Biol.* 75: 297-317.

72. Wendt, I.R. and Gibbs, C.L. (1973). Energy production of rat extensor digitorum longus muscle. *Am. J. Physiol.* 224: 1081-1086.

73. Yang, W.C.T., Geiger, P.J., Bessman, S.P. and Borrebaek, B. (1977). Formation of creatine phosphate from creatine and ^{32}P-labelled ATP by isolated rabbit heart mitochondria. *Biochem. Biophys. Res. Commun.* 76: 882-888.