This is to certify that the

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Energy Metabolism in Skeletal Muscle:
Effects of ATP Depletion

presented by

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ENERGY METABOLISM IN SKELETAL MUSCLE: EFFECTS OF ATP DEPLETION

By

Jeanne Marie Foley

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY

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ABSTRACT

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The specific problem addressed by this research concerned the validity of a kinetic theory of control of oxidative phosphorylation rate by amount of the phosphate acceptor ADP in skeletal muscle. Respiratory control by ADP concentration requires that, for a given rate, any reduction in ATP must be countered by a compensatory increase in the ratio Cr/PCr in order to maintain the same ADP level, assuming equilibrium of the creatine kinase reaction and no large increase in pH. This prediction was tested by depleting ATP in rat gastrocnemius muscles and using phosphorus NMR to monitor ATP, PCr, P_i and pH during rest and contraction in situ. ADP was calculated from these parameters and the creatine kinase equilibrium constant.

ATP was depleted by combining intense stimulation with blockage of the purine nucleotide cycle (PNC) with the specific inhibitor hadacidin (N-formyl-N-hydroxyaminoacetic acid) to prevent resynthesis of adenine nucleotides from inosine monophosphate. Initial experiments using the drug AICAr (5-amino-4-imidazolecarboxamide riboside) demonstrated systemic effects of this drug, countering previous claims that muscle dysfunction associated with AICAr infusion was attributable to selective PNC inhibition.

When hadacidin was used to sustain ATP depletion, the decrease in PCr predicted by the kinetic control theory did not occur; in fact, the

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Cr/PCr ratio actually dropped slightly in resting ATP depleted muscles relative to controls, and pH decreased slightly. Calculated ADP levels also rose much less in response to a series of isometric twitch contractions in ATP-depleted muscles than in control muscles subjected to twitch stimulation at the same rate. Calculated phosphorylation potential ($\ln\{[ATP]/([ADP]*[P_i])\}$) was identical in resting control and ATP-depleted muscles, and the decline in this parameter was only mildly attenuated in depleted muscles during submaximal stimulation. These results suggest that phosphorylation potential, rather than ADP alone, is the cytoplasmic factor which regulates respiration in muscle cells. Other effects observed in association with ATP depletion included an apparent reduction in the energy cost of both twitch and tetanic contractions and a potentiation of peak force in successive trains of tetani.

This dissertation is dedicated to four people whose affection and support has sustained and encouraged me throughout this work and to whom I warmly acknowledge a lifetime debt of personal appreciation.

To Sax, my best (and oldest!) friend.

To Curt and Judy, for helping me keep my sanity and sense of perspective over these last five years.

And to Jane, for having the patience of Sarah. (me, too!)

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I. INTRODUCTION

"In the muscle, nature has produced a machine, so startling and at the same time so perfect, that the explanation of its mechanism could give satisfaction not only to the searching mind, but also promise a rich harvest to the technical progress of mankind."

Otto Meyerhof, 1925 (115)

Muscle energy metabolism and its regulation have been the subjects of intense research and debate throughout the history of physiological investigation. As early as 1880, a regulatory link was proposed between the mechanics of contraction and the underlying muscle chemistry (153). During the century following this speculation, the details of muscle structure, mechanics, and chemistry were brought into focus. The question of the precise nature of the signal linking metabolic energy production with contractile energy demand has, however, remained incompletely resolved.

The advent of nuclear magnetic resonance (NMR) spectroscopy and the burgeoning application of this technology to physiological studies during the past decade have generated new insights into this lingering question of metabolic regulation. The NMR method allows accurate tracking of the changing levels of several energetically important intracellular metabolites during contraction and recovery in living muscles. Although much of this information could also be compiled by traditional chemical methods, the NMR technique offers the advantage of noninvasive study of ongoing metabolic processes in an intracellular environment that is virtually undisturbed, in contrast to the traumatic disruption required for conventional methods. The NMR method also allows serial measurements within a single muscle, thereby improving

practically obtainable time resolution and increasing statistical power over the one-muscle-one-time-point restriction of the older methods. In addition, the new technology provides some novel capabilities, reviewed in the following chapter, that were out of reach of previously existing methods.

The studies described in chapters three and four supplemented traditional chemical methods with the newer spectroscopic techniques to study the effects of experimental manipulation of muscle adenine nucleotide levels on metabolic responses to contraction. The general aim of this research was to examine the effects of adenosine triphosphate (ATP) depletion on energy metabolism in skeletal muscle. The specific question being addressed was: Is kinetic control (by amount of the phosphate acceptor ADP) of the rate of oxidative phosphorylation sufficient to explain this regulation in contracting skeletal muscle? The existence of the creatine kinase reaction in a near-equilibrium state in muscle (103) provided the specific hypothesis to be tested.

The concentration of ADP can be estimated from the levels of the other reactants and products of the creatine kinase reaction and from the equilibrium constant (K_{eq}) of the reaction according to the equation (146):

$$[ADP] = [ATP]^* ([Cr]/[PCr]) * (1/[H^+]) * 1/K_{eq}$$

If [ADP] controls respiration rate, then for a given rate any reduction in ATP must be countered by a compensatory increase in the ratio Cr/PCr in order to maintain the same ADP level, assuming no large change in pH. This prediction was tested by depleting ATP in rat gastrocnemius muscles and using phosphorus NMR to follow the changes in ATP, PCr and pH

during rest and contraction in situ.

Review of the methods by which ATP depletion had been induced in previous studies led to the choice of a technique combining intense stimulation to deplete ATP with blockage of the purine nucleotide cycle (PNC) with the inhibitor hadacidin to prevent resynthesis of adenine nucleotides from inosine monophosphate. As reviewed in detail in the following chapter, this method was selected because it introduced the fewest confounding factors into the experimental analysis.

Before these studies could begin, however, it was necessary to address the recent claim (42) that PNC inhibition per se impairs aerobic energy metabolism in muscle. Further review of the literature revealed, however, that the drug AICAr used in this study is known to affect other enzymes outside of the PNC in vitro. AICAr treatment had also been associated with systemic effects in vivo, most notably hemodynamic abnormalities. Furthermore, previous studies with the PNC inhibitor hadacidin had demonstrated no adverse effects of PNC inhibition on either submaximal twitch or intense tetanic contraction capacity (113).

This information led to the formulation of the hypothesis that the administration of AICAr caused systemic effects and that these systemic effects, not PNC inhibition, were the basis of the performance decrement observed in the study cited above. To test this hypothesis the cited experiments were repeated while trying to unmask any possible systemic effects by monitoring arterial blood pressure with an indwelling catheter and phosphorus metabolites and pH with ^{31}P-NMR. The hypothesis was then tested directly by surgically isolating and artificially perfusing cat muscles with an AICAr perfusate. If the aerobic performance decline was indeed due to systemic hemodynamic

effects, then isolation of the muscle from the cardiovascular system in this preparation should eliminate these performance effects.

The outcome of these experiments was the detection of profound suppression of blood pressure in AICAr-infused rats and failure to recover PCr stores after a twitch series. In the isolated, perfused muscles, there was no adverse effect of AICAr on twitch force, and PCr recovered normally following the contraction series. Therefore it was concluded that PNC inhibition per se does not adversely affect muscle aerobic metabolism ((46), chapter three).

The specificity for PNC inhibition of the drug proposed for use in our initial research plan has been amply demonstrated, as documented in chapter two. The findings of our initial investigation cleared the way for moving forward with these studies to test the kinetic respiratory control hypothesis described above. An infusion/stimulation/recovery protocol was developed to consistently produce depletion of nearly half of the normal ATP stores in the rat gastrocnemius muscle in situ. The decrease in PCr predicted by the kinetic control theory did not occur; in fact, the Cr/PCr ratio actually dropped slightly in resting ATP depleted muscles relative to controls. Calculated ADP levels also rose much less in response to a series of isometric twitch contractions in ATP-depleted muscles than in control muscles subjected to twitch stimulation at the same rate. These results indicate that mitochondrial respiratory rate control cannot be satisfactorily explained solely on the basis of kinetic mechanisms, assuming that respective respiration rates at rest and during the stimulation period did not differ substantially between the two groups.

These studies also produced several additional, unanticipated

outcomes. First, the energy cost of twitch and tetanic contractions appeared to be reduced in the ATP-depleted muscles relative to controls, complicating the interpretation of the ADP changes observed in response to stimulation. Secondly, tetanic stimulation caused the accumulation of markedly more acid in control than in ATP-depleted muscles, suggesting a reduction in the rates of glycolysis and glycogenolysis in the depleted muscles. Finally, ATP depletion was associated with a staircase or potentiation of peak force during series of tetanic contractions. This phenomenon was observed in both control and hadacidin-treated muscles when subjected to intermittent tetanic stimulation immediately following an initial intense stimulation to deplete ATP. This staircase effect disappeared after recovery of ATP in the control group, but persisted in the muscles whose ATP recovery was blocked.

The rationale for the development of these studies and the implications of the results are best considered in the context of the existing body of knowledge in this area. To this purpose, an historical review of the research on muscle energy metabolism and its regulation is presented in the following chapter.

II. REVIEW OF LITERATURE

"The extreme simplicity of the fabric (of muscle) ... has been the cause of the obscurity that prevails in understanding how a small, soft, fleshy portion can produce such strong and ample motions."

Baron Albertus Haller, 1786 (54)

The 'fabric' of muscle and the mechanisms underlying the activity of muscle have been subjects of intense scrutiny throughout the history of physiological inquiry. In the mid 1700's, William Croone proposed that muscle was the biological material upon which studies would be most likely to yield an understanding of "the energy discharges of living elements" (44). As the eighteenth century drew to a close, however, the "motive cause" of muscle action was still attributed to "a law immediately derived from God" (54). Technological advances during the 1800's spawned more sophisticated studies of the mechanisms of contraction and led to the development of the concept of muscle as a chemical machine.

EARLY HISTORY OF MUSCLE ENERGY METABOLISM RESEARCH: FROM LACTATE TO ATP

In the late 19th century, Hermann and later Pflüger conceived of a complex "lactacidogen" or "inogen" molecule, containing lactic acid and made irritable by the inclusion of oxygen. Anabolism was thus viewed as the formation of "elaborate, unstable, oxygen-charged molecules" into an "irritable protoplasm" which could then be discharged as needed to provide energy for life processes (44).

This view was challenged by Fletcher and Hopkins, who were able to

demonstrate that the immediate supply of oxygen affected contraction (44). The inogen theory, based as it was on the *prior* inclusion of oxygen, was thus rendered unlikely. These two researchers also pioneered a new low-temperature method of killing and extracting muscles to minimize excess lactic acid production caused by the excision and extraction procedures themselves. They noted that, prior to their 1907 work using the new technique, "there is hardly any important fact concerning lactic acid formation in muscle which, advanced by one observer, has not been contradicted by another" (45). The work reported in this paper by Fletcher and Hopkins was later hailed by Meyerhof as "the first bridge between the biochemistry of muscle and its performance of work" (115).

The Lactate Hypothesis

> *"In the evolution of muscle it would appear that advantage, so to speak, has been taken of this acid phase in carbohydrate degradation, and that by appropriate arrangement of the cell elements the lactic acid, before it leaves the tissue in final combustion, is assigned the particular position in which it can induce those tension changes upon which all the wonders of the animal world depend."*
>
> Fletcher and Hopkins, 1917 (44)

Fletcher and Hopkins authored this "lactate hypothesis", wherein the hydrogen ions from lactic acid supposedly interacted directly with the myofibrillar surface. Lactate was thus viewed as the direct cause of increased tension, leading to the notion that lactate was "part of the machinery and not part of the fuel." Fatigue and rigor were seen as manifestations of lactate accumulation: "...fatigue is the expression, not of an exhaustion of the energy supply, but only of a clogging of the machine." Oxygen's purported role was to 'unclog' the

machine by removing lactic acid (44).

In 1921, Hartree and Hill noted that the temperature coefficient of the rate of heat liberation from a muscular contraction was of the same magnitude as those of ordinary chemical reactions. This led them to conclude that muscle energy supply regulation in a prolonged contraction is a chemical process (58). In this report they also outlined a regulatory scheme in which a 'shock' or nerve impulse caused permeabilization of the walls of a lactic acid compartment within the muscle cell, thereby releasing lactate onto the fibrillar surface to directly cause increased tension in contractile structures. According to this model, relaxation was caused by neutralization or other means of removal of the acid from its site of action. The role of oxygen was to facilitate this relaxation process, either by oxidizing lactate or by somehow causing it to be returned to the impermeable intracellular compartment. Although this theory seems preposterous in retrospect, at the time it was proposed it very nicely conformed to and explained the known facts.

Hartree and Hill also related the mechanical efficiency (defined here as work done per unit of heat liberated per second of stimulus) of muscle to its 'quickness', observing that unstriated muscles exhibited a very high efficiency in long term maintenance of force. They proceeded yet further in characterizing fast vs. slow muscles, stating

> *"...it is well known to athletes that heavy and exhausting exercise is very bad training for sports, such as sprint-running and jumping, where great quickness is required: the training which leads to quickness of contraction and relaxation is directly opposed to that which leads to economy, and immunity from fatigue, in slow, prolonged, and heavy movements." (58).*

Thus was the principle of specificity of training, a basic tenet of modern exercise physiology, initially and most eloquently recorded.

In 1924, a student of A.V. Hill reported a series of painstaking experiments demonstrating that muscle has the ability to vary its energy transformation rate according to the tension production and duration of a contraction (35). This phenomenon, which became known after its author as the 'Fenn effect', initiated a search that continues even today for the signal linking energy demand to production.

Muscle Buffer Capacity Examined

Later the same year, Hartree and Hill again collaborated on a paper, this time concerning the apparent need for a large buffer capacity in muscle. With deft calculations using existing data, they concluded that a mere ten seconds of 'violent effort' by a human athlete would produce enough lactic acid to increase hydrogen ion concentration 18 fold. Given the buffers then known to be available to muscle, this would produce a pH of 5.75, lower than had ever been observed even in isolated muscles. The authors exclaimed that "it is difficult to imagine that such a change in cH (*sic*) in the intact animal could be tolerated without disaster" (59). The experimental section of this article reported the results of the addition to an isolated muscle of an amount of exogenous lactic acid approximating that expected from a ten second maximal contraction. The observation that intracellular [H^+] increased only by a factor of 2.2 (i.e. pH decreased to 6.7) prompted the authors to conclude that some heretofore unidentified but highly effective buffer must exist in muscle. According to the chemical data available at that time, they speculated that this unknown substance was probably an "alkaline protein buffer".

The question of acid neutralization in muscle was further

addressed in 1925 by the Australian Tiegs. Although Hartree and Hill and earlier Meyerhof had claimed that the neutralizing base must be a muscle protein, Tiegs dissented. He proposed that the buffering base was actually produced simultaneously with the lactic acid (144). This view, though closer to the actual truth, seemed based on rather unconvincing histological evidence.

Tiegs also surmised a link between the buffer production process and the compound creatine, noting that creatine content in muscles varied in proportion to their activity. Citing previous work, he remarked that "plain" (smooth) muscles were lowest in creatine content and "quickly contracting 'pale' muscles" (fast white skeletal muscles) were highest. Again citing earlier evidence that creatine increased with stimulation, Tiegs offered three possible mechanisms by which the "very feebly basic" creatine could undergo conversion to a base of sufficient strength to neutralize the lactate produced during contraction. The first two routes, conversion to the strong bases creatinine or dimethylguanidine, were ruled out by showing that these compounds were not present in stimulated muscles. Thus remained only the third of Tiegs' alternatives, namely that creatine was transformed to a more basic isomer upon stimulation of muscle.

To accommodate this hypothesis, Tiegs suggested that the 'normal' form of creatine in resting muscle was not the commonly-held open structure, but rather a cyclic form. Upon stimulation, this cyclic molecule would undergo conversion to "active creatine" by opening of the ring structure to expose a free amino group, thus providing the necessary increase in effective base. Although his experiments on isolated frog muscle did confirm that creatine was indeed liberated from

exercising muscles, his explanation of the mechanism of increased buffer capacity was clearly contrary to the known chemistry of that time.

The 'lactacidogen' theory which Tiegs held as "now quite certain" was decisively refuted by Meyerhof, also in 1925. The theory was based in large part on the claim of a lactic acid maximum due to a limited amount of precursor. Meyerhof showed that lactic acid production by isolated frog gastrocnemius could be increased to 50% beyond the supposed maximum by speeding removal of lactate from the muscle via added alkali in the bath (115). Despite this caveat, Meyerhof still subscribed to the 'lactate hypothesis' in which lactate directly caused the contractile event.

The Discovery of Phosphagen

Experiments conducted at Harvard University and at University College in London during the period 1925-1927 finally shifted the muscle energy focus away from lactic acid and towards organic phosphorus compounds, generating a "revolution in muscle physiology" as it was subsequently designated by A. V. Hill (60). In 1927, the American team of Cyrus Fiske and Yellapragada Subbarow and, working independently, the Britons Grace and Philip Eggleton separately reported the discovery of a labile organic phosphate compound whose stimulation-induced degradation released inorganic phosphate inside muscle cells.

Most historical accounts of muscle metabolism research provide only a scant mention of this discovery. Most such essays also give primary credit for phosphocreatine (PCr) discovery to the Eggletons, despite the fact that Fiske and Subbarow were not only the first to correctly identify the labile phosphorus compound as phosphocreatine, but were also much more accurate in their speculations as to its

functions. For these reasons, the circumstances surrounding this breakthrough will be dealt with in some detail here.

In 1925, Fiske and Subbarow published a detailed account of a new colorimetric assay for inorganic phosphate (P_i) in biological samples. Their primary improvement over the older Briggs method was the substitution of an alternative reducing agent which reduced the time to maximal color development from 30 minutes to less than five minutes (41). Following up on apparently erroneous results they obtained using this new method, the pair discovered that previous estimates of muscle inorganic phosphate content were mistakenly high due to excess orthophosphate liberated during the extraction and assay process. Since this excess phosphate release was virtually complete within 30 minutes, the longer color development time required by the older assay method tended to mask the orthophosphate increase. In 1927, Fiske and Subbarow decisively and correctly identified the labile organic precursor in a terse abstract in the Journal of Biological Chemistry, given here in its entirety:

"Only a small fraction of what has previously been regarded as inorganic phosphate in voluntary muscle is actually inorganic. The bulk of it is an unstable compound of creatine and phosphoric acid which is hydrolyzed upon stimulation and resynthesized when the muscle is permitted to recover." (40)

In April of the same year, a more detailed account of their findings appeared in the American journal Science. In this article, Fiske and Subbarow showed that the "delayed color development" of their 1925 P_i assay when applied to muscle tissue was due to a "labile phosphorus" compound (38). The amount of unstable phosphorus was estimated from successive color intensity readings at short intervals until full color development. Extrapolation back to zero time (muscle

excision) gave true 'inorganic' phosphate content, the difference between this and the maximal value being 'labile' phosphorus. They identified this precursor of P_i as a derivative of creatine (Cr). They also observed that pre-stimulation of the muscle prior to extraction and assay reduced the labile phosphorus by two-thirds compared with resting muscle. Additional experiments demonstrated recovery of the phosphorus-creatine compound in stimulated muscle that had been rested briefly prior to excision. The authors remarked that the release of P_i and Cr by fatigued muscles, previously attributed to fatigue-induced alteration of membrane permeability, could now be explained simply by increased concentration gradients of the two compounds resulting from stimulation-induced hydrolysis of PCr inside the muscle cell. Thus their discovery not only introduced a new compound into the muscle energy scheme, but also began to weaken the underpinnings of the lactate hypothesis by casting doubt upon the reality of the permeability changes required in the Hartree/Hill model of contractile regulation (58).

Regarding their observation of delayed color development and their suspicion that an unstable organic phosphorus compound existed in muscle, Fiske and Subbarow commented,

> *"Although these facts have been in our possession now for more than a year, we have until this time refrained from placing them on record, inasmuch as the phenomena observed could not with any certainty be ascribed to the presence of an organic compound of phosphoric acid until the compound had been isolated, or at least until the organic radicle (sic) had been identified." (38)*

This commitment to presentation of a complete story unfortunately was to cost these two men much of their deserved credit for this landmark discovery.

In February of 1927, after submission but before publication of

the Fiske and Subbarow report, there appeared in the Biochemical Journal an article by Grace and Philip Eggleton announcing the discovery in muscle of an unstable organic phosphorus compound which they designated as 'phosphagen' (30). The Eggletons used the 1922 Briggs method for determining P_i, taking color intensity readings at two minute intervals for 30 minutes rather than a single reading at 30 minutes as had been done in previous applications of the method. The Eggletons noted that color development in P_i standards and in extracts of dead muscle was exponential with a time constant independent of the phosphorus concentration. In extracts of fresh muscle, however, this time constant was much larger. Therefore, they concluded, previous estimations of muscle inorganic phosphorus were erroneously high due to the acid hydrolysis of an unstable organic phosphorus compound during the color development period.

Using a clever extrapolation procedure, they estimated that the 90-100 mg of 'inorganic phosphate' per 100 g of muscle reported earlier by Briggs, Embden and others actually consisted of only 20-25 mg P_i, the remainder being 'phosphagen'. They also noticed that the amount of phosphagen was lower in fatigued muscles than fresh, and practically nonexistent in muscles in rigor.

The Eggletons speculated that this 'phosphagen' might be a phosphate ester of glycogen, or perhaps a combination of lactate and phosphoric acid, i.e. the 'lactacidogen' impugned by Meyerhof. In a subsequent letter to the British journal Nature, the Eggletons stated, "Whilst we have here at present no definite knowledge of the nature of this substance, it seems quite possible that it may be the unstable ('active') hexose monophosphate..." (32). Their next paper claimed that

"recent unpublished work on the isolation of phosphagen shows that it is a hexosemonophosphoric acid, though some doubt attaches to the nature of the hexose" (32). Based on this result, the authors proposed a reaction scheme in which phosphagen split to yield lactate and P_i , the latter recombining with glycogen to give a compound 'X' which "may be identical with lactacidogen". The final reaction of this proposed cycle regenerated phosphagen from 'X'. Summarizing this model, the Eggletons remarked, "These three reactions form a cycle of changes which, if properly balanced, leads simply to the conversion of glycogen into lactic acid." The proposal of this scheme can be viewed as a testimonial to the prevalence of the lactate hypothesis, inasmuch as it attempted to fit the new discovery into the lactate-centered model rather than recognizing the new compound as the key to an entirely new explanation of muscle energy generation.

In February of 1928, the 'special articles' section of Science carried another succinct report by Fiske and Subbarow entitled "The Isolation and Function of Phosphocreatine" (37). This communication provided the correct empirical formula and chemical structure of PCr isolated from rabbit muscle. In what can certainly be in retrospect deemed a major understatement, the authors commented, "... the instability of the phosphamic group marks it as one of considerable biological importance".

Fiske and Subbarow additionally observed that phosphocreatine was not only hydrolyzed during activity, but also resynthesized during subsequent recovery. According to their calculations, the P_i liberated by PCr hydrolysis during stimulation was sufficient to neutralize much of the lactic acid produced. This prompted their assertion that

"... the function of phosphocreatine in muscle -- or one function, since there may be others -- (is) that of neutralizing a considerable part of the lactic acid formed during muscular contraction." This fulfilled the 1924 prediction of Hartree and Hill regarding the existence of a novel, highly effective buffer in muscle (59).

Later in 1928, the Eggletons reported some "Further Observations on Phosphagen" (29), in which they described the isolation of a labile compound of creatine and phosphoric acid, finally acknowledging the Harvard research by a footnote: "Confirming Fiske and Subbarow". The British team still maintained, however, that "... it is not yet certain whether this substance is identical with, or is a breakdown product of phosphagen".

The sharp contrast between the previously noted commitment of Fiske and Subbarow to present a finished research picture and the Eggletons' approach to publication is illustrated by the introduction to the 1928 Eggleton article:

"The collection of experimental results presented in this paper is too inconsecutive to be used as the basis of any theoretical discussion of phosphagen, but the results are published in the hope that they will be of practical use to other workers in the field of muscle chemistry." (29)

Without reference to their previous misidentification of phosphagen as a hexose phosphate and likely precursor of lactic acid, the Eggletons acknowledged in this paper that phospagen breakdown and lactic acid production were chemically distinct mechanisms.

One of the 'inconsecutive' results of this study that did prove to be of importance later was the correlation of rate of energy output with muscle phosphagen content. This relationship had been suggested in one of their earlier papers (32), based upon the observation that

gastrocnemius muscle contained more phosphagen than did cardiac muscle, and smooth muscle contained little or none (31). This correlation was presaged by Tiegs' earlier observation that muscle creatine content varied with muscle activity (144). The 1928 Eggleton report compared the phosphagen:P_i ratio in various skeletal muscles, noting a higher ratio in the "quicker" muscles, such as the gastrocnemius, than in "muscles intended for lower rates of energy expenditure", such as the soleus (29).

The same paper reported the results of a comparative study in which the absence of phosphagen and creatine and the prevalence of arginine were noted in invertebrate muscle. In an insightful commentary, the Eggletons observed that both arginine and creatine gave the same "colour reaction" characteristic of a guanidino group and argued that this could be the basis for physiological function in both.

The following year, now nearly two years after the identification of phosphocreatine by Fiske and Subbarow, the Eggletons produced yet another paper in which they claimed that "The exact nature of phosphagen is not yet known" (28). They advised that the term 'phosphagen' be retained "for the substance existing in muscle", as distinguished from the PCr isolated from muscle extracts. They went on to conclude that "... the 'breakdown' and 'resynthesis' of phosphagen in a living muscle are reactions involving very little energy". Later in this paper they reported their failure to replicate some published results of Fiske and Subbarow, provided that "clean glassware and pure reagents are used".

Recognition Controversy

In view of the preceding account, the position accorded the Eggletons in most historical records as the discoverers of phosphocreatine in muscle seems unmerited, particularly since Fiske and Subbarow have received only secondary credit or mere passing mention in most muscle metabolism reviews. Fiske and Subbarow were not only the first to isolate, purify, and identify phosphocreatine in muscle; they also proved more meticulous in their methods and much more accurate in their deductions of PCr function in muscle than did the more acclaimed Eggletons.

Fiske and Subbarow showed evidence of their awareness of this controversy in an article in the 1929 Journal of Biological Chemistry. In this 50-page discourse, the two physiologists directly addressed the claims of the Eggletons in a most pointed manner:

> *"Eggleton and Eggleton, using the Briggs method -- which we have shown to be inaccurate -- likewise have observed the slow development of color in the case of filtrates from ... muscle ... These authors, however, also without experimental evidence, chose the alternative assumption, viz., that the cause of the phenomenon is the presence of an unstable organic compound of phosphoric acid, and hazarded the guess that they might be dealing with a new variety of hexosemonophosphate, or 'a phosphoric acid ester of glycogen.' Somewhat later, in a paper published several months after we had announced that the labile phosphorus in muscle is combined with 1 equivalent of creatine, Eggleton and Eggleton claimed to have shown that muscle contains a hexosemonophosphate with the properties that have been described above, though admitting that 'some doubt attaches to the nature of the hexose.' As a fitting accompaniment to this extraordinary assertion, for which they could not possibly have had the slightest evidence, Eggleton and Eggleton offered an elaborate hypothesis in which the attempt was made to incorporate this hexosemonophosphate into the series of chemical reactions (involving glycogen, lactic acid, etc.) already known to occur during muscular contraction. Needless to say the theory, based as it was on a structure devoid of facts, is valueless, and has since been quietly abandoned by its authors."* (39)

The remainder of this extensive article was devoted primarily to

an exhaustive account of the extraction, purification, and assay methods used in obtaining the results reported in the two years prior. This paper represented the last published work by Fiske and Subbarow on the topic of phosphocreatine. Nothing further appeared over Fiske's signature until a 1935 study of 'depressor substance' (adenosine) in the blood, followed in the 1940's by studies devoted to purine chemistry. Likewise, Subbarow produced no additional publications until the late 1930's, when he reappeared as a secondary author on several studies involving nutrition, vitamins, and growth factors.

The Eggletons' perceived place in history was most likely the result of a 1932 article by the eminent British physiologist A.V. Hill. In the opening statements of this widely acclaimed review entitled "The Revolution in Muscle Physiology", the influential Hill gave full credit for the breakthrough discovery to the Eggletons:

"The revolution to which this paper refers broke out on the last day of December, 1926, when Eggleton and Eggleton sent to the Biochemical Journal a paper describing phosphagen, a labile form of organic phosphate in muscle. In 4½ years their discovery has changed our outlook on the relation between chemistry and the energy exchanges of muscle ..." (60)

Several pages later in the paper appeared the sole mention of Fiske and Subbarow, a brief paragraph remarking that "Shortly after, and independently of, the Eggletons' publication, Fiske and Subbarow reported the same discovery ..." Hill went on to downplay the role of the Harvard team in deducing the buffering functions of phosphocreatine by claiming that this conclusion had been suggested in the 1925 Tiegs article (144). It should be recalled that Tiegs had maintained that creatine *itself* provided the extra buffering power by conversion to a more basic isomer upon stimulation; the existence of a phosphorus-creatine compound, much less its hydrolysis to release buffer phosphate,

was not even remotely suggested in this paper.

This apparent oversight in an otherwise comprehensive synthesis of studies could perhaps be considered a reflection of Hill's position as a collaborator and colleague of the Eggletons at University College. Alternatively, Hill's apparent disregard of Fiske and Subbarow's work might be viewed as a reaction to their 1929 "Phosphocreatine" paper (39) in which they harshly criticized the work of not only the Eggletons, but also other European scientists such as Embden, Lohmann, and Meyerhof. In any case, the outcome of Hill's pronouncement has been that later reviews of muscle energy metabolism concepts have tended to give primary credit for the discovery of phosphocreatine to the Eggletons (e.g. Lundsgaard (98), Lohmann (93, 94), Huxley (69), Hill (64), Lipmann (91)). A notable exception is the monograph by Kalckar (72) who, perhaps not coincidentally, hailed from Harvard.

Demise of the Lactate Hypothesis

Credit controversy notwithstanding, the discovery of the presence, hydrolysis, and resynthesis of phosphocreatine in muscle did indeed lead to a 'revolution' in muscle physiology by finally turning the research focus away from lactate as the key chemical in muscular contraction. By 1930, Einar Lundsgaard had proposed a system of linked reservoirs in which 'phosphagen' was the final energy source for muscular contraction (99). In Lundsgaard's model, the phosphagen pool was fed in turn by a glycolytic reservoir involving lactate formation. He also envisioned a third "carbohydrate oxidation reservoir" which could either lead to lactate formation or directly augment the phosphagen reservoir without coincident lactate production. This model is now recognizable as the

first reasonable approximation of the modern paradigm of the muscle energy system. Lundsgaard was particularly prescient in his conception of the third, oxidative reservoir, since the citric acid cycle and electron transport system had not yet been proposed.

The key experimental evidence leading to the development of this model consisted of the demonstration by Lundsgaard of "alactacid" contractions in muscles treated with iodoacetic acid (IAA) to block glycolysis. Achievement of these results required an ingenious experimental design, since animals treated with IAA normally develop rigor in all skeletal muscles shortly following treatment. Lundsgaard discovered, however, that prior paralysis of a limb via nerve section served to protect the muscles of that limb from rigor development. He had previously observed that the rigid muscles of unparalyzed, IAA treated frogs contained no acid in excess of that normally found in untreated, resting muscles; this was in great contrast to the large lactate accumulation normally associated with rigor development.

Pursuing this clue, Lundsgaard electrically stimulated his IAA treated, denervated muscles and observed series of twitches in which no lactate was produced. Furthermore, the poisoned muscles fatigued faster than normal muscles, and exhaustion coincided with the near total depletion of phosphagen. This demonstration finally and conclusively struck down the lactate hypothesis by clearly dissociating lactate production from contraction, and lactate accumulation from fatigue.

The linked reservoir model was suggested to Lundsgaard by two facts: unpoisoned muscles performing the same number of twitches as IAA-treated muscles retained 70-75% of their phosphagen stores, and the energy liberation from the extensive PCr hydrolysis in poisoned muscles

approximated the combined energy of PCr hydrolysis and lactate formation in unpoisoned controls. From this evidence he deduced that lactate formation normally provided energy for the post-contraction resynthesis of the phosphagen utilized in contraction. This solved Hill's persistent problem of the source of the "delayed anaerobic heat" following muscular contraction. Lundsgaard also commented that the previous failure of researchers to correlate PCr breakdown with contractile work output was now most certainly due to the confounding effects of glycolysis.

Although Lundsgaard's scheme represented major progress, his model still placed PCr in the position of direct energy source for contraction. He was certainly aware of the existence of ATP, or "adenylpyrophosphosäure" as it was named by its discoverer Lohmann in 1929 (92). However, Lundsgaard's observation that no appreciable ATP breakdown occurred until development of exhaustion and rigor misled him to conclude that PCr, not ATP, must be the direct source of energy for contraction. In 1932, Hill lauded Lundsgaard's work as "admirable in expression and execution", but in the same paper Hill prophetically mused, "I wonder whether we are still failing to see something which in 10 years will seem obvious" (60).

The ATP Challenge

The missing piece to the muscle energy puzzle was indeed provided during the ensuing decade. In 1934, Karl Lohmann reported that PCr hydrolysis in muscle extracts occurred only in the presence of ATP and ATPase, whereas ATP hydrolysis was able to proceed even when PCr breakdown was inhibited by alkaline pH (94). He further noted that ATP exerted a catalytic influence on PCr hydrolysis in muscle extracts, with

one part ATP, in the presence of ATPase, capable of causing the splitting of 1000 parts PCr by "creatine-phosphoric acid-splitting enzyme".

Lohmann proposed the following scheme, which became known as the 'Lohmannn reaction' (93):

(A) $\quad ATP \langle\!=\!=\!=\rangle AMP + 2P_i$

(B) $\quad AMP + 2\ PCr \langle\!=\!=\!=\rangle ATP + 2\ Cr$

(C) (Net) $\quad 2PCr \langle\!=\!=\!=\rangle 2Cr + 2\ P_i$

Although this model showed ATPase directly hydrolyzing ATP to AMP without the intermediate myokinase reaction and portrayed AMP rather than ADP as the phosphate acceptor in the creatine kinase reaction, Lohmann did correctly infer that ATP hydrolysis was the direct energy-supplying reaction in muscle contraction. He was able to explain Lundsgaard's observation that muscle ATP levels do not fall significantly until exhaustion (99) by showing that the creatine kinase reaction (B) proceeds much faster than the ATPase step (A). Therefore, Lohmann argued, PCr hydrolysis rapidly regenerated ATP so that little "adenylsäure" (AMP) built up until PCr stores began to fail.

Although Lohmann's proposal constituted the missing "something" of Hill's earlier speculation, it would be nearly 30 years before the presentation of decisive proof that ATP was indeed hydrolyzed during contraction of intact muscles. Throughout the 1930's and 40's, physiologists continued to argue against Lohmann's theory, citing the undeniable fact that all supporting experimental evidence was derived from chemistry experiments on muscle extracts rather than from studies

of intact muscle.

A 1937 paper by Sacks, Sacks, and Shaw was representative of the resistance to Lohmann's concept. In this article, the first to report on muscle contracting in a steady state of twitch force, the authors confirmed Lundsgaard's earlier observation (99) that ATP apparently did not undergo decomposition unless muscle was stimulated at a relatively high twitch rate until fatigued (131). Furthermore, this study demonstrated that although ATP was degraded under such circumstances, lactic acid also accumulated in large amounts. The authors reasoned, somewhat speciously, that these results were inconsistent with Lohmann's claim that ATP synthesis occurs at the expense of lactate formation.

Despite lingering opposition, evidence began to mount in support of Lohmann's claim that ATP was the energy source closest to muscular contraction. Needham deduced this fact from a series of observations beginning with the apparently unique character of "adenylpyrophosphatase" (ATPase) in muscle:

> *"Other phosphatases in muscle are, so far as it is known, very feeble and unimportant...Now experiments on muscle extract indicate that no enzyme is present for splitting creatinephosphoric acid into creatine and free phosphate, but only for catalysing its reaction with adenylic acid... If adenylpyrophospatase is really the only muscle phosphatase, it is clear that the phosphate must have come from this source..." (122)*

Additional suggestive evidence surfaced in 1939, when Englehardt and Ljubimowa established that muscle ATPase activity was so intimately intertwined with myosin as to be inseparable from it by any standard fractionation method (33). Hydrolysis of ATP was thus at least enzymatically possible at a site directly involved in the contractile process.

In 1941, Fritz Lipmann proposed a novel chemical mechanism by

which phosphagen might store metabolic energy to be utilized not only for muscular contraction, but for many other cell processes as well (90). In this remarkable paper, Lipmann introduced the terms 'high-energy phosphate bond' and 'group potential' and the symbol ~ph (later rendered as ~P). Despite the major advance represented by this idea, Lipmann persisted in identifying PCr as the primary direct energy source for contraction, hypothesizing that, " Pyrophosphatase (ATPase) operates rather like an outlet for the adenylic acid system to adjust the flow of ~ph in case of overproduction, much in the manner of a valve."

Despite Lipmann's adherence to the notion of the primacy of PCr, Lohmann's earlier theory specifying ATP as the direct energy source for contraction gradually gained acceptance during the decade following Lipmann's phosphate energy transfer proposal. Lohmann's chemical experiments on muscle extracts (93, 94) were repeated by numerous others, and the results confirmed his reaction rate reports. This evidence was bolstered by the previously noted localization of ATPase activity to the contractile apparatus and by the apparent character of ATPase as the sole quantitative phosphatase in muscle.

Eventually the growing body of chemical evidence convinced even skeptics such as Lundsgaard, who finally abandoned his earlier position (99), admitting that, "Most likely inorganic phosphate does not originate directly from phosphocreatine but from adenosinetriphosphate" (98).

Acceptance aside, the fact still remained that ATP hydrolysis in intact muscle had not yet been demonstrated during individual contractions or submaximal twitch series. This persistent doubt was rekindled by the venerable A. V. Hill in a 1949 letter to Nature (61).

Although Hill conceded the *likelihood* that "the energy of contraction is derived in the first instance from the breaking of the terminal energy-rich phosphate bond of ATP", he contended that the issue could not be finally laid to rest until ATP hydrolysis could be conclusively associated with contraction/relaxation rather than recovery. In this letter, Hill also offered some possible experimental approaches to achieving this goal. He advocated working at lower temperatures to slow reaction rates, and using tortoise muscles, noting that "... frogs' and rabbits' muscles are singularly ill-suited to the enquiry, they are much too quick ..."

Hill elaborated on this theme the following year in his famous "Challenge to Biochemists", published in a special issue of Biochemica et Biophysica Acta commemorating the 65th birthday of Otto Meyerhof. In this essay, Hill spoke of the new 'revolution' in muscle physiology in which ATP hydrolysis seized the role of the "fundamental change", supplanting the phosphagen which had engendered the revolution out of the "lactic acid era". Hill commented:

"It may very well be the case, and none will be happier than I to be quit of revolutions, that the breakdown of ATP really is responsible for contraction or relaxation: but in fact there is no direct evidence that it is." (62)

Hill went on to speak somewhat disparagingly of in vitro work on muscle extracts, indicating his strong bias toward working on living muscle: "And I when I say muscle, I mean muscle: living muscle". Expanding on the suggestions advanced in his 1949 commentary (61), Hill put forth some ideas for new techniques involving metabolic poisons, different animal species, and other variations of method:

"If one's instruments, or methods, are too slow, one can make them relatively quicker by using slower materials -- tortoises, toads

or even sloths. That means, of course, that biochemists, like biophysicists, must also be biologists (as Meyerhof always has been and as Hopkins was) -- but why not?" (62)

Continuing on this topic later that same year, Hill stated, "Indirect evidence suggests that ATP occupies a key position in the chemical machinery of contraction; but it remains possible that its intervention is confined to the chemical process of recovery." At this point, Hill conceded that even the modified chemical methods he had suggested earlier might be too slow and insensitive to resolve the question. As an alternative, he promoted the use of physical methods, particularly heat measurements. Lundsgaard (97) pointed out that labeled-phosphate experiments had verified that "ATP constantly is broken down and rebuilt in the intact muscle", although he did admit that it had not been shown that the rate of ~P turnover in ATP was increased by stimulation.

At a symposium chaired by Hill on the physical and chemical basis of muscular contraction, the Cambridge biochemist Dorothy Needham proposed a novel method for approaching the ATP 'challenge'. Crediting Herman Kalckar for development of the chemical techniques, Needham outlined in detail an 'enzymatic spectrophotometric' method for following ADP production rather than ATP loss for determining the extent of ATP breakdown during a single contraction or short series of twitches (121). The method depended upon a clever series of enzymatic conversions of the ADP released by ATP hydrolysis into a compound with a UV absorption maximum sufficiently distant from that of ATP so as to be differentiable from ATP even in minuscule quantities. Needham recognized that such a method held "The great advantage that it aims at estimating a small increase from zero concentration", in contrast to

attempts to measure a very small decrease in the relatively large muscle ATP concentration. Thus her method addressed the first of the two shortcomings, sensitivity and speed, of previous chemical methods as identified by Hill.

Needham was, however, clearly aware of the limitations of her proposed method, noting that its success depended upon the ability to instantly (and reproducibly) arrest the cellular enzymes at the height of the twitch and to minimize the effect of the "traumatic stimulus" on ATP breakdown. She also conceded that the rate of the creatine kinase reaction in vivo was unknown, and might exceed the time resolution capacity of the chemical methods.

A variation of this experimental design was employed by Mommaerts and Rupp, who reported their results in a letter to Nature in late 1951 (119). It is interesting to note that, although their methods adhered closely to the proposal of Needham, these authors failed to acknowledge her contribution even though their awareness of her work was evident from their citation of Hill's introduction to the symposium in which it was presented.

In any case, Mommaerts and Rupp claimed to have shown using these methods that ATP was clearly hydrolyzed during contraction. However, close examination of their work reveals some serious problems which, taken together, render their results inconclusive. First of all, application of simple statistical methods to the analysis of the raw data presented in this article shows that, although the ADP values showed a *tendency* to increase in the contracted muscle, this difference was not statistically significant (one way ANOVA, $p > .05$). Secondly, their assay methods were incapable of separating the unbound,

physiologically reactive fraction of the total ADP from the larger, bound portion. Furthermore, they did not measure PCr and therefore could not address Needham's concern (121) regarding the extent of rephosphorylation of ADP. Finally, their results suggested that "precipitous cooling mobilizes the contractile apparatus to a somewhat greater extent than occurs physiologically in a single twitch." Such an interaction between contraction and the freezing process would serve to reduce yet further the true contraction-induced difference in ADP levels between the control and stimulated muscle groups.

The challenge posed by Hill in 1950 would stand without an ultimate solution for more than 10 years after Mommaert and Rupp's efforts. Finally in 1962, Robert Davies and his student D. F. Cain reported the results of a study using the recently discovered CK inhibitor 1-flouro-2,4-dinitrobenzene (FDNB), which had been shown by Kuby and Mahowald to completely inhibit creatine kinase in vitro (76). Previous work from Davies' University of Pennsylvania laboratory, including Cain's 1960 doctoral dissertation, had provided the first conclusive evidence that PCr was hydrolyzed during a single contraction. Using the new inhibitor to block PCr breakdown, Cain and Davies were now able to finally and irrefutably demonstrate that ATP itself was also degraded within a single twitch (13). According to their results from isolated, FDNB-treated frog muscles, 0.44 μmol/g muscle of ATP was hydrolyzed per twitch. This figure agreed nicely with the 0.5 μmol/g value predicted on the basis of an assumed thermodynamic efficiency (81) of 50% for the isotonic workload performed. The in vivo inhibition of CK by FDNB was confirmed by the fact that the PCr content of these stimulated muscles was the same as in resting, untreated controls.

Furthermore, the importance of phosphagen's role in energy metabolism was illustrated by the 90% reduction in number of twitches produced before onset of fatigue.

At last Hill had his proof, in "muscle: living muscle" that ATP hydrolysis was not confined to the recovery process of muscular contraction.

BEYOND ATP: DEVELOPMENT OF CONCEPTS REGARDING ENERGY USE AND REPLENISHMENT

The discovery of PCr in 1927 and of ATP in 1929, along with the 1934 proposal by Lohmann correctly identifying their relative positions in muscle energy metabolism, set the stage for the development of modern concepts of muscle physiology. The next three decades brought elucidation of the oxidative and glycolytic mechanisms by which the high energy phosphate pools are maintained and replenished, as well as precise characterization of the molecular mechanism by which ATP hydrolysis drives muscular contraction. These discoveries have been well chronicled; their treatment here will be limited to a brief overview and citation of pertinent original studies and reviews.

The Citric Acid Cycle

The regeneration of phosphagen in stimulated muscles allowed to recover in oxygen was described in the earliest reports on "labile phosphorus" (37, 38). A large step toward clarification of the mechanism for this oxidative regeneration of energy was made by Krebs and Johnson in the now famous 1937 Enzymologia paper, in which "experiments are reported which ... allow us to outline the principle steps of the oxidation of sugar in animal tissues" (74). In developing

the concept of the tricarboxylic acid (TCA) cycle or Krebs cycle, as it came to be known, the authors keyed on the catalytic nature of the effect of citrate on respiration. Added citrate was observed to increase oxygen consumption, and this effect was "by far greater than can be accounted for by the complete oxidation of citrate." The enhancement of respiration was even more pronounced if additional carbohydrate was added, prompting the researchers to "presume therefore that the substrate the oxidation of which is catalyzed by citrate, is a carbohydrate or related substance."

In an excellent integration of their results with previous reports by other researchers, Krebs and Johnson proposed a cycle involving citrate, malate, fumarate, oxaloacetate, succinate and α-ketoglutarate. Clever application of metabolic inhibitors illustrated the likely relative positions of the elements in this cyclic pathway. In addressing the question "from which substance the two additional carbon atoms of the citric acid molecule are derived", the authors predicted that the answer was to be found in a triose derivative such as pyruvate or acetate.

The Electron Transport System.

Kreb's citric acid cycle explained the mechanism of oxidation of the 2-carbon breakdown products of carbohydrate precursors, and of lipid substrate as well, but the connection between oxidation of foodstuffs and phosphorylation of ADP and Cr remained to be drawn. Finally in 1956, Britton Chance and G. R. Williams synthesized decades of results from a variety of sources into a coherent model joining oxidation of the "dihydrodiphosphopyridine nucleotide" (DPNH, or nicotinamide adenine

dinucleotide (NADH)) produced by the TCA cycle to mitochondrial ADP phosphorylation (16).

Citing the work on oxidases and cytochromes by Warburg, Keilin and others, Chance and Williams outlined the components of the electron transport system (ETS) and their likely relative positions in "the respiratory chain, the main pathway for the transfer of electrons or protons from metabolites to oxygen." The authors recognized that:

> *"Work along distinctly different lines has shown at least three properties of the respiratory chain to be of fundamental importance in the metabolic and regulatory functions of the cell; (1) to transfer electrons or protons from substrates to oxygen and particularly to maintain the necessary level of oxidized DPN (NAD^+) within the aerobic cell; (2) to act as a sequence of three or more energy conservation steps by which ADP is converted to ATP so that the latter is available as a common medium for energy expenditure throughout the cell; and (3) to regulate the metabolism in accordance with the levels of control substances, for example, of ADP itself or of a hormone upon the rate or efficiency of the energy conservation process." (16)*

In addition to this characterization of the ETS, Chance and Williams reviewed the work on uncouplers of phosphorylation from oxidation in an attempt to uncover the mechanism of the coupling of the ETS to ATPase activity. The results of these uncoupling studies led the University of Pennsylvania pair into " ... postulation of new and presently unidentified high-energy intermediates which participate in the oxidative phosphorylation process." This interpretation, however, was to be rendered invalid by the work reported five years later by Mitchell.

The Chance and Williams paper also reported extensively on their own work regarding steady states of respiration in isolated mitochondria. From these results, the authors defined five metabolic states of mitochondria, each characterized by a distinct combination of substrate level, ADP level, respiration rate, and oxidation levels of

NAD and of specific ETS components. The experimental significance of these states was considered to be their utility as tools for examining "the nature of components of the respiratory chain involved in oxidative phosphorylation."

The remainder of this exceptional paper utilized work on transitions between the defined metabolic states of isolated mitochondria to formulate a theory of respiratory control. Chance and Williams proposed that control was achieved by reversal of an inhibition of respiration rather than by direct activation. The authors concluded that "ADP itself (is) probably the physiological substance responsible for the activation of respiration of biological systems following stimulation." The evidence supporting this conclusion was, however, based almost exclusively on the results of experiments involving the addition of excess ADP to suspensions of isolated liver mitochondria. As A.V. Hill might have cautioned, the direct application of the resulting model to intact, living muscle was perhaps premature.

Chance and Williams did concede that "Several workers have suggested that the ATP/ADP ratio should determine the respiration rate of the mitochondria, but our experiments provide evidence that this is not the case." Curiously, none of the three papers cited as exemplifying the work supporting this opposing view did in fact directly address the issue of ATP/ADP as controller. Siekevitz and Potter reported on the establishment of experimental conditions in isolated mitochondria in which respiration rate increased despite lack of change in either ADP or ATP (125). These authors went on to suggest that ATP, ADP, and P_i all together "are able to regulate the oxidative rate in the mitochondria according to physiological need." They did

not, however, propose any mechanism by which such control might occur.

The Lardy and Wellman paper cited by Chance and Williams also proved devoid of arguments for respiratory control by ATP/ADP ratio, although it did claim that "In general, rates of respiration vary inversely with the '~P potential' against which the oxidative system must work (86)". The third paper offered by Chance and Williams as typical of this opposing view in fact dealt with oxidative phosphorylation in insect sarcosomes and primarily recounted methods of isolation and inhibition. No mention of respiratory control by either ADP or ATP/ADP was made, excepting this brief notation in an appendix: "Except at very low concentrations of ADP, (the rate of synthesis of ATP by oxidative phosphorylation) is independent of the concentration of ADP (89)".

Even though these cited sources did not provide worthy examples of the exceptions taken to Chance and Williams' theory of respiratory control by level of phosphate acceptor, controversy regarding the nature of this control was indeed spirited. This debate persists even today, and provides one of the questions addressed by the research reported in later chapters of the present work. Details of alternative views of respiratory control are presented later in this chapter.

Despite some continuing skepticism regarding the Chance/Williams concept of respiratory control, their work did undoubtedly greatly advance the understanding of electron transfer and oxidative pathways of energy production. A key issue remaining unsatisfactorily explained, however, was the question of how oxidation was linked to phosphorylation of ADP.

The Chemiosmotic Hypothesis.

In 1961, Peter Mitchell proposed a novel mechanism whereby phosphorylation could be accomplished by either oxidative or photosynthetic electron transfer (116). This new concept was based on a completely different approach than the orthodox view as championed by Chance and Williams, which postulated the existence of a 'high-energy intermediate' in order to reconcile oxidative phosphorylation with the known mechanism of substrate-level phosphorylation in glycolysis. In addition to the fact that these chemical intermediates were "elusive to identification", Mitchell listed a number of other shortcomings and inconsistencies of the chemical model. Among these points of contention were the failure of the prevailing model to explain the close association of phosphorylation with membranous structures or the shrinking/swelling phenomena associated with phosphorylation activity, and the fact that oxidation could be uncoupled from phosphorylation at three different sites and by agents possessing "no identifiable specific chemical characteristic."

To explain these inconsistencies, Mitchell proposed an entirely new mechanism based on the concept of group translocation:

> *"This type of mechanism differs fundamentally from the orthodox view in that it depends absolutely on a supramolecular organization of the enzyme systems concerned. Such supramolecularly organized systems can exhibit what I have called chemi-osmotic coupling because the driving force on a given chemical reaction can be due to the spatially directed channeling of the diffusion of a chemical component or group along a pathway specified in space by the physical organization of the system." (116)*

The three basic features of Mitchell's model were a reversible ATPase system, an oxido-reduction system for transferring electrons and protons, and a charge-impermeable membrane across which these two systems

would be anisotropically located. The electron/proton translocator was proposed to create a proton gradient across this membrane. The resulting electrochemical potential would then provide the driving force for phosphorylation of ADP via a channeling mechanism resident in the ATPase component. This model yielded a number of testable predictions which withstood subsequent experimentation, as reviewed in 1979 by Mitchell in a lecture delivered on the occasion of his acceptance of the Nobel prize for chemistry (117).

In summarizing the metabolic symmetry inherent in his new model, Mitchell concluded his original paper as follows:

"In the exact sciences, cause and effect are no more than events linked in sequence. Biochemists now generally accept the idea that metabolism is the cause of membrane transport. The underlying thesis of the hypothesis put forward here is that if the processes that we call cell metabolism and transport represent events in a sequence, not only can metabolism be the cause of transport, but also transport can be the cause of metabolism. Thus we might be inclined to recognize that transport and metabolism, as usually understood by biochemists, may be conceived advantageously as different aspects of one and the same process of vectorial metabolism." (116)

Muscle Structure, Mechanics, and Energetics.

The molecular mechanism by which ATP hydrolysis drives muscular contraction was laid out in some detail by Davies in a 1963 treatise in Nature (22). Acknowledging the contributions of numerous other scientists, including Huxley's sliding filament hypothesis (development reviewed by its author (69)), Davies presented a molecular theory based on calcium-dependent interactions of "interdigitating filaments" of actin and myosin. The role of ATP hydrolysis in the development of tension was that of breaking the electrostatic bonds of actin-myosin crossbridges to permit "quantal contractions".

Muscle studies throughout the remainder of the 1960's and 70's

dealt in large part with the long list of predictions enumerated by Davies in this paper. The results of the physical and chemical studies confirming and expanding upon the contractile energetics theory advanced by Davies have been recently reviewed in detail by Kushmerick (80).

Application of phosphorus NMR Spectroscopy to Muscle Studies.

"It is usually open to the physicist or pure chemist to control and simplify the conditions of his experimental work, or wisely to avoid regions of complexity until collateral progress has made them simple. In biology the complexities of the conditions are in the essence of the phenomena, and the experimentalist, when he tries to simplify them, is even viewed with suspicion. Thus even the operation of excising a muscle before studying its chemistry has been regarded with some prejudice..."

Fletcher and Hopkins, 1915 (44)

Until the mid-1970's, studies of metabolite changes in working muscle required sampling of discrete time points by the process of freezing, excision, and acid extraction of the muscles and completion of an individual chemical assay for each metabolite to be studied. With the development of biological applications of phosphorus nuclear magnetic resonance (^{31}P-NMR) spectroscopy, continuous monitoring of the intracellular levels of the energetically important metabolites ATP, PCr and P_i could be accomplished simultaneously in tissues and even in intact muscles in living animals and in human subjects. The words of A.V. Hill in heralding the development of the galvonometer at the turn of the century find a new and fitting application to the effect of NMR technology on modern-day muscle research: "(This new technology) has rendered fertile again a field of work which in (previous) days seemed barren by reason of poorness of methods" (63).

The physiochemical phenomenon of NMR was first reported in 1946 by

Bloch (9) and Purcell (127), who shared the 1952 Nobel physics prize for their independent achievement of this discovery. Their work provided a new analytical tool for chemists, allowing the use of radio waves and magnetic fields to nondestructively study the structure and composition of chemical compounds. These initial chemical applications exploited the fact that a hydrogen nucleus placed in a strong magnetic field resonates between specific quantum energy states whose separation varies with the chemical environment surrounding that nucleus as well as with the strength of the applied field. Therefore a proton in a given molecular environment in a static magnetic field will absorb and emit radiofrequency energy of only the specific frequency corresponding to the energy quantum separating the resonance states. Each chemically distinct proton thus exhibits a characteristic resonance frequency by which the chemical entity within which that proton lies can be identified.

Biological applications of this method surfaced with the refinement of NMR techniques directed toward the phosphorus nucleus. This method allowed the measurement of relative levels of phosphorus compounds present in millimolar amounts within the target sample. In muscle tissue, this includes the high energy phosphate compounds ATP and PCr, as well as inorganic phosphate but few other peaks to complicate the frequency spectrum.

The first application of ^{31}P-NMR to living cells was reported by Moon and Richards in a 1973 study of 2,3-diphosphoglycerate levels in erythrocytes (120). The following year Hoult, et al, published the first ^{31}P-NMR study of intact tissues (154). Descriptive studies of phosphorus metabolite levels in intact muscles were reported in 1976 by

Burt, Glonek, and Bàràny (11, 12). The first experimental studies of muscle contraction and recovery were communicated by Wilkie's group in 1977 (24), and scores of muscle studies followed.

Entirely noninvasive studies of specific muscles became possible with the development of the surface coil technique by Ackerman, et al, in 1980 (1). Prior to this breakthrough, the restriction to a saddle-type coil configuration required excision or at least surgical isolation of the muscle for placement within the coil cylinder, or cross sectional studies of entire limbs affixed within such a coil.

Although the muscle research field could not be properly characterized as "barren" prior to the introduction of this new method, the NMR technique does offer many advantages over classical chemical methods. The new method is not without its own drawbacks, however. Disadvantages of the NMR technique include the high cost and limited availability of the superconducting magnets, specialized probes, and computer systems required to collect and analyze NMR data, as well as the technical difficulties attendant upon operating equipment near such strong magnetic fields. The aforementioned sensitivity limitation precludes the direct measurement of metabolites present at concentrations much below 1 mM. Data analysis is also complicated by the need to standardize relative peak areas to some known concentration value in order to convert spectral integrals into absolute concentration units. The actual process of integrating the peak areas in the frequency spectrum also poses interpretative problems which have been addressed (109) but not yet entirely resolved. In surface coil experiments, care must be taken in defining the location of the muscle volume from which the NMR signal arises (83). The standardization and

other technical difficulties have been sufficiently resolved to permit widespread use of the NMR technique for in vivo muscle studies (see Meyer (103) for review) but some caution must still be taken in evaluating and interpreting NMR studies in view of the analytical methods applied.

Of primary importance among the advantages offered by the NMR method is the noninvasive nature of the technique. NMR studies provide information arising from intact cells in a physiologically realistic setting, whereas classical chemical methods require gross disruption of the cellular membrane and substructures in preparation of the muscle sample for analysis. This shortcoming of the traditional method has long been recognized and was aptly described by Fletcher and Hopkins in 1917:

> *"The inherent difficulty besetting the chemical examination of muscle lies, of course, in the fact that the necessary processes for extraction of the constituents cause in the moment of their application profound chemical change."* (44)

Needham also recognized early on the effect of the "traumatic stimulus" of the freezing process on muscle phosphagen levels (121); the interaction of this stimulus with the contraction process itself has already been noted as a confounding factor in earlier experiments (119).

Another major advantage of the NMR method is that it allows monitoring of metabolite levels at multiple time points before, during and after contraction of a single muscle. In contrast, the conventional method provides but one time point per animal. In addition to the obvious increase in practically obtainable time resolution and reduction in number of experimental animals required, this feature of the NMR method also substantially reduces statistical variability by allowing

comparison between successive time points within the same subject rather than the between subject comparison to which the classical method was limited. One practical consequence of this fact is that a muscle can be used as its own control in some NMR experimental designs (e.g. 107, 2).

Time resolution on the order of 15-30 seconds is easily obtainable with existing NMR equipment and methods (e.g. 107, 101). Precision of timing can be increased yet further via gating methods which synchronize data collection to a cyclical event such as repetitive electrical stimulation of a muscle (24, 2).

The NMR method also provides a very sensitive, accurate probe of intracellular pH, as demonstrated by the first biological application of phosphorus NMR (120). Because the resonant frequency of a peak in an NMR spectrum shifts with the association or dissociation of hydrogen ions from the molecule generating that signal, the position of the peak corresponding to a weak acid or base reflects the pH of the solution in which that compound resides. In ^{31}P-NMR spectra from muscle tissue, the position of the inorganic phosphate peak ($pK_a \approx 6.8$) provides a very useful, noninvasive indicator of intracellular pH within the normal physiological range (83). Furthermore, the NMR method permits the *simultaneous* measurement of pH and phosphorus metabolite concentrations.

Although the free, metabolically active quantity of ADP in muscle cells is well below the threshold for ^{31}P-NMR detection in vivo (103), the free ADP concentration can be estimated from NMR-measurable parameters assuming equilibrium of the creatine kinase reaction (83). Chemical assays of ADP in muscle extracts measure a total ADP value on the order of 1 mM, but this amount includes the large fraction of ADP that is bound tightly to macromolecules such as F-actin and is thus

metabolically unreactive. The free fraction of ADP represents less than 5% of this total (146). Estimation of free ADP via the CK equilibrium can, of course, be done using metabolite values obtained by traditional chemical methods (146), but such calculations generally overestimate [ADP] because of inflation of Cr values and reduction of PCr values (103) due to artifactual hydrolysis as noted above.

Finally, use of magnetization transfer techniques allow direct measurement of the rates of exchange in chemical reactions. Whereas biochemical methods permit such measurements only in cell-free systems attempting to approximate intracellular conditions, the NMR method allows exchange rate analysis to be done within living cells in an intact organism (10).

Numerous and extensive reviews have been made of the large body of metabolic information acquired over the past two decades using the NMR technique on living muscle. These include general reviews on NMR principles and biological applications (e.g. 49, 10), as well as reviews of specific areas such as applications to general tissue metabolism (151), metabolic control principles (15), and striated muscle metabolism (103).

CURRENT CONCEPTS IN MUSCLE ENERGY METABOLISM

The modern muscle energy scheme incorporates all of the discoveries outlined in the previous sections. In this system, the direct source of energy for muscular contraction is ATP. Hydrolysis of ATP via actomyosin ATPase (reaction A below) provides energy to drive crossbridge movements resulting in sarcomere shortening. The immediate resource for regenerating the ATP pool is phosphocreatine, which acts via creatine kinase to rephosphorylate ATP (reaction B). The effectiveness of the creatine kinase system is such that, at all but the most intense workloads, ATP levels are maintained at an essentially constant level. The net reaction observed is therefore the hydrolysis of PCr (reaction C), as noted by Lohmann in 1934 (93):

(A) $\quad ATP \quad \langle\!=\!=\!=\!=\rangle \quad ADP + P_i \quad$ (ATPase)

(B) $\quad PCr + ADP \quad \langle\!=\!=\!=\!=\rangle \quad Cr + ATP \quad$ (creatine kinase)

(C) (Net): $\quad PCr \quad \langle\!=\!=\!=\!=\rangle \quad Cr + P_i$

The total muscle phosphagen stores available vary somewhat by species and fiber type, but average roughly 5-8 mM for ATP and 20-30 mM for PCr in mammalian skeletal muscle (e.g. 107, 83; see Table 1). Assuming an energy cost of 0.2 μmol ~P/g muscle/twitch (68), the entire ATP/PCr pool would provide enough energy for roughly 100 twitches. Unassisted, this pool could support the most intense workloads for only a few seconds, or a mild workload for only a minute or two even if the entire pool could be hydrolyzed. Augmentation of these immediate energy sources is accomplished by oxidative phosphorylation, involving the mitochondrial respiratory chain and mitochondrial ATPase and creatine

TABLE 1. Typical metabolite concentrations (μmol/g) in selected mammalian muscles at rest.

Muscle	P_i	PCr	Cr	ATP	free ADP
rat gastroc in situ (83)	3	27	4	7.5	0.02
cat biceps isolated, perfused (107)	3	35	0.2	9	0.0003
cat soleus isolated, perfused (107)	10	17	8	5	0.014

kinase enzymes, and/or the cytosolic pathways of glycolysis/ glycogenolysis. The mechanisms by which the cell matches the rate of oxidative phosphorylation with changing ATP demand in working muscle has been studied extensively, but no firm consensus has yet been reached regarding the correct model of respiratory control. The experiments in this study have been designed to provide further information for discriminating between some of the proposed mechanisms outlined below.

Control of Respiration

The mass action ratio $([ADP]*[P_i])/[ATP]$ for reaction A above is maintained at a steady state level far from equilibrium in the vicinity of myofibrils. In resting muscle, the reciprocal of this quantity, termed the phosphorylation state, is on the order of 1×10^6 M^{-1} (higher in fast, glycolytic fibers; lower in slow, oxidative fibers) (146). This compares to an expected value at equilibrium of 1.5×10^{-5}. Consequently, a chemical potential of about 60 kJ/mole is available for conversion to mechanical work upon activation of the actomyosin ATPase.

Reaction B, on the other hand, is poised near equilibrium in muscle cells (103). Therefore the creatine kinase system can respond to any decrease in ATP/increase in ADP with a corresponding change in Cr/PCr. Response is very rapid because of the high catalytic efficiency and high muscle levels of creatine kinase, and very sensitive because of the steady state levels of the adenine nucleotides (see Table 1). In fast twitch muscle, for example, hydrolysis of just 1/10 of 1% of ATP can cause nearly a 10 fold increase in ADP, resulting in a 90% drop in the ATP/ADP ratio.

As mentioned above, the CK system on its own could serve to maintain ATP levels only briefly, particularly since it is apparently impossible for living cells to completely drain their phosphagen pools (143). The process of regeneration of ATP must then be supplemented by mitochondrial oxidative phosphorylation and/or anaerobic glycolysis and substrate-level phosphorylation. The question arises: what is the signal that transmits the myofibrillar energy need to the mitochondrial respiratory system?

The simplest theory of respiratory control is perhaps the model in which availability of ADP to the mitochondrial ATP synthetase determines the rate of oxidative phosphorylation. According to this view, the rate of ATP synthesis would depend on substrate concentration in classic Michaelis-Menten fashion. This model, proposed by Chance in 1956 as noted previously, appears adequate to describe respiratory control in isolated mitochondria and has in fact been accepted by many as the mechanism operating in intact muscle. Indeed, this mechanism of respiratory control is presented as fact in many current biochemistry texts (e.g. 398). Some adherents of this kinetic theory postulate that

it is actually the diffusion of creatine from myofibrillar to mitochondrial creatine kinase that carries the [ADP] information. This 'creatine shuttle' hypothesis (8) is examined in greater detail in the next section.

Alternatively it has been argued that mitochondrial respiration is responsive to the thermodynamic entity $[ATP]/([ADP]*[P_i])$ (80). Although classical thermodynamics does not permit the prediction of reaction rates from concentration ratios, postulation of a near-equilibrium network of the reactions of oxidative phosphorylation and cytosolic energy metabolism allows use of non-equilibrium thermodynamic principles to provide a time factor link (14). According to this theory, metabolite ratios from other reactions in this network, most notably oxidation of mitochondrial NADH, will also affect the rate of rephosphorylation of ATP (73).

Fluctuating intracellular calcium levels have also been implicated in respiratory regulation. Elevated free cytosolic Ca^{++} levels link excitation of the muscle cell with crossbridge formation, ATP splitting, and contraction of the myofibrils. This 'calcium signal' can also affect the activity of enzymes such as mitochondrial dehydrogenases (56), thereby altering the rates of citric acid cycle flux and the mitochondrial redox potential. Glycogen phosphorylase kinase activity is also increased by elevated Ca^{++}, thus providing a link between contraction activation and glycogenolysis (100).

Functional Role of the CK System

The most directly apparent function of PCr in muscle is to provide an energy store for maintenance of high ATP levels during periods of increased ATP turnover. This 'temporal buffering' effect is the result of thermodynamic considerations as previously discussed.

It has also been posited that the CK system provides an essential diffusive link, or 'creatine shuttle' of ~P, between myofibrillar and mitochondrial adenine nucleotide pools. According to this view, the flux of ATP/ADP between these two compartments is limited by some diffusion barrier. Thus PCr/Cr diffusion is proposed to provide a "spatial buffer" between these two compartments and their localized CK and ATPases (8).

A mathematical model developed by Meyer, Sweeney, and Kushmerick (112) demonstrated that the transport (spatial buffering) and classical (temporal) buffering functions of the CK system both could arise simply from the near-equilibrium state of the CK reaction. Physical compartmentation is therefore not required to explain the transport function of PCr/Cr. Thus the CK system is apparently not required for diffusive transport of ~P, but it can significantly increase the rate of this transport, thereby maintaining a given rate of diffusive flux with smaller spatial gradients of ATP and ADP than would otherwise be possible. This lessens the dissipation of the free energy of hydrolysis of ATP that would occur if the adenine nucleotides themselves carried all of the diffusive flux. The model equations demonstrated that this 'spatial buffering' aspect of the CK system would likely be important only in relatively large cells with a low density of mitochondria (e.g. fast, glycolytic fibers).

In addition to energy buffer functions, the presence of a large store of PCr in muscle cells also allows the generation of a much larger increase in free inorganic phosphate for a given net drop in ATP. This feature of the CK system has several interesting implications. First of all, if the ATPase reaction occurred in isolation, a 1/10% decline in ATP would produce a decrease of about 89% in [ATP]/[ADP] and 90% in [ATP]/[ADP]*[P_i] as per the previous example using fast muscle metabolite parameters. With the creatine kinase system in place, however, even a very moderate workload might easily cause a 30% decline in PCr. In the fast muscle example under consideration, this amount of PCr hydrolysis could raise P_i 10-fold or more, resulting in a correspondingly larger reduction in [ATP]/[ADP]*[P_i] than would have been possible without the CK system. This 10-fold amplification of the thermodynamic signal could provide much finer control of respiratory rate if the near-equilibrium network theory is correct.

The ability to generate large increases in P_i via the CK system could also affect metabolic regulation in several other ways. The CK reaction produces the divalent anion $HPO4^{--}$, with a pK_a of 6.8. At an intracellular pH of 7.0, this divalent P_i will take up protons until the ratio of mono- to di-protonated species reaches 1.6 ($10^{(7-6.8)}$). Thus for every mole of P_i produced via the CK reaction, 0.4 moles (1/(1+1.6)) of protons are consumed. The resulting drop in [H^+] may be enough to relieve H^+ inhibition of PFK, thus accelerating the rate of glycolysis (80).

The presence of this additional free phosphate also increases the effective buffer capacity of working muscle, as first noted by Fiske and Subbarow over 60 years ago (37). This increase serves to attenuate the

intracellular acidification that would otherwise occur when a high rate of anaerobic glycolysis causes significant production of lactic acid. Although numerous studies have demonstrated a correlation between intracellular acidification and fatigue (e.g. 132), more recent experiments have produced evidence indicating that it is diprotonated P_i rather than acidification per se that mediates fatigue (52, 152), perhaps by directly inhibiting the actomyosin ATPase complex (23). This is consistent with an earlier report that experimentally lowering intracellular pH to levels comparable to those produced by intense workloads did not significantly affect muscle force, provided that P_i was kept low (111). Recent NMR studies confirmed this dissociation of fatigue from acid accumulation, and also demonstrated fatigue generation independent of $H_2PO_4^-$ levels (3).

It has also been suggested that increased phosphate levels might induce Ca^{++} sequestration within sarcoplasmic reticulum (and mitochondria) by formation of a calcium phosphate precipitate within the organelle (75). This trapping of calcium could reduce the amount of excitation-induced calcium release, thereby reducing the contractile response.

Circuit model.

"(Oxygen) is used presumably in some process whereby the molecular machinery of the muscle carries out oxidations for the storing of 'free energy'... analogous to the oxidation of coal in a steam engine in order to turn a dynamo and so to charge an accumulator -- whereby free energy, the power of carrying out life-processes, is stored in the living tissue, which power, which free energy, may be utilised by the tissue whenever an appropriate stimulus is applied."

A.V. Hill, 1913 (63)

Although the analogy suggested by Hill in 1913 referred to "the storing of potential energy ... in the conversion of lactic acid into glycogen" (115) according to the lactacidogen theory in vogue at the time, the "accumulator" concept was resurrected in later years and applied to the high energy phosphate scheme of muscle energetics. The first such application was outlined by Lipmann in 1941. He proposed a model based on an electric motor, in which phosphocreatine served as a "~P-accumulator", ATP as the "wiring system", and the oxidation-reduction system as the "metabolic dynamo" (90). This model was only used, however, as a schematic for illustrating "the machine-like functioning of the revolving sequence of reactions", and not as a tool for predicting the behavior of the system.

Nearly half a century later, the qualitative and quantitative behavior of some aspects of muscle oxidative metabolism have now been successfully modeled by a simple electrical circuit analog (104). In this model, the creatine kinase system functions as a chemical capacitance (Fig. 1). PCr is represented by the charge accumulated on the capacitor, with capacitance (C) proportional to the total creatine level (T_c). The resistance component R_m depends on density and functional properties of the mitochondria and varies inversely with the oxidative capacity of the muscle fiber. The cytosolic free energy of hydrolysis of ATP is modeled as V_o, and the mitochondrial value as the battery V_b. Current flow through circuit models the rate of hydrolysis/resynthesis of ATP or the flow of energy in the form of 'high-energy' phosphate (~P). The ability of the CK system to temporally buffer ATP levels means that a work-to-rest transition, modeled as a step change in cytosolic ATPase rate, results in a new

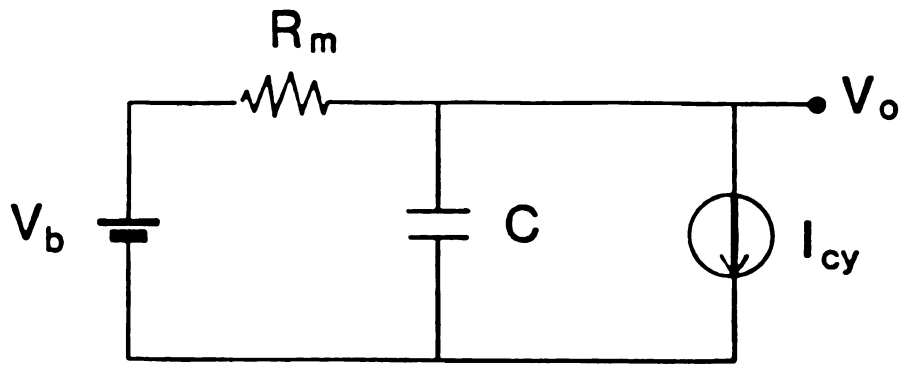

FIGURE 1. Electrical circuit model.

Key: V_o = cytoplasmic phosphorylation potential
V_b = mitochondrial potential
C = capacitance due to CK system
R_m = mitochondrial resistance term
I_{cy} = cytoplasmic ATPase activity (flow of ~P)
I_{Rm} = mitochondrial ATP production rate

(From Meyer (104))

steady state level of cytosolic free energy of hydrolysis not far from the original value of Vo.

The temporal changes in PCr as modeled by this system will be monoexponential, with time constant for the RC circuit given by $\tau=R_m*C$. Thus the model predicts that the time constant for PCr changes during transitions from one steady state (e.g. rest) to another (e.g. some submaximal contractile rate) will be proportional to the total creatine level for a given resistance value. According to this model, decreases or increases in total creatine content should result in respectively shorter or longer time constants for PCr changes in response to initiation or termination of a bout of moderate exercise, provided there is no change in the mitochondrial resistance value.

The circuit-analog prediction that PCr time constants ought to vary linearly with total creatine content was tested recently in a study

utilizing the creatine analog β-GPA to induce Cr/PCr depletion in muscle cells (101). Since β-GPA-P is hydrolyzed only very slowly during submaximal muscle work, the replacement of Cr/PCr by analog results in reduction of the effective total creatine (T_c) pool. Utilizing feeding periods of 2, 4, 6, and 8 weeks, T_c was reduced in rat gastrocnemius muscle by amounts ranging from 40 to 90%. ^{31}P-NMR was used to follow PCr changes during stimulation at three submaximal (aerobic) rates and during subsequent recovery.

The results show that PCr time constants did indeed scale linearly with T_c. This finding provides evidence against the creatine shuttle hypothesis, since a loss of Cr/PCr would result in larger spatial gradients of ATP and ADP and a substantial drop in free energy of ATP if normal creatine content was a requirement for maintenance of normal ~P flux. This decreased deltaG(ATP) would be modeled as an increase in R_m in the circuit model, thus countering the effect of a decreased T_c on PCr time constants.

This RC circuit model can also be used to estimate the rate of energy use at the onset of stimulation. Utilizing the capacitative circuit analogy, the level of phosphocreatine remaining at time t after the onset of stimulation is given by

$$PCr(t) = PCr_{ss} + (PCr_o - PCr_{ss})e^{-t/\tau}$$

where PCr_{ss} = steady state PCr concentration, PCr_o = initial PCr level, and time constant $\tau = R_m * C$ as noted earlier. The time rate of change of PCr is then given by

$$\frac{dPCr}{dt} = -\frac{1}{\tau}(PCr_o - PCr_{ss})e^{-t/\tau}$$

The energy use in the first seconds of contraction will be reflected almost entirely by PCr hydrolysis, since glycolytic and oxidative mechanisms do not immediately contribute significantly to energy production (155). Therefore the value of the function dPCr/dt at t=0 should provide a good estimate of the initial energy cost of contraction. This application is used in the interpretation of experimental results in the fourth chapter of this paper.

EXPERIMENTAL MANIPULATION OF ADENINE NUCLEOTIDES

One approach to resolving the issue of kinetic ([ADP]) vs. thermodynamic ([ATP]/([ADP]*[P_i])) control of oxidative phosphorylation rate is to study the effects of controlled alterations of these parameters in vivo. Muscle ATP can be depleted by ischemia (20, 27, 48) or by iodoacetate inhibition of glycolysis (25, 47), but these manipulations produce, respectively, structural damage or irreversible alteration of metabolic function and hence are unsuitable methods for respiratory control studies. Very intense work is also known to deplete ATP, but this effect is relatively short-lived (143). ATP stores depleted by heavy work can be prevented from recovering to normal levels, however, by blocking the purine nucleotide cycle (PNC). In rapidly contracting muscle there will be significant net hydrolysis of ATP to ADP, which in turn is converted to AMP via myokinase. AMP then enters the following series of reactions which constitute the purine nucleotide cycle (reviewed by Lowenstein (95)):

(A). AMP + H2O ====> IMP + NH3 (AMP deaminase)

(B). IMP + asp + GTP ====> sAMP + GDP + P_i (sAMP synthetase)

(C). sAMP ====> AMP + fumarate (sAMP lyase)

Experiments by Meyer and Terjung (113, 108) used the drug hadacidin (N-formyl-hydroxyaminoacetic acid; biochemistry reviewed by Shigeura (135)) to inhibit the PNC at step B. Hadacidin has been shown by others (87) to produce very specific inhibition of sAMP synthetase. Infusion of the drug followed by intense stimulation was found to cause prolonged depletion of ATP levels by up to 50% in rat gastrocnemius (fast) muscle. PNC inhibition had no adverse effects on muscle performance at submaximal workloads, although the effect of the hadacidin-induced ATP depletion on subsequent work performance was not examined in these studies.

In contrast to these results, recent reports of studies using the sAMP lyase inhibitor AICAr (5-amino-4-imidazolecarboxamide riboside; pharmacology reviewed by Hano (55)) indicated an apparent disruption of aerobic muscle function attributed by the authors to non-replenishment of Kreb's cycle intermediates due to blockage of fumarate production at PNC step C (43). However, earlier reports of AICAr interaction with myriad other enzyme systems (e.g. 6, 55, 79) weakened the assumption of PNC specificity upon which this conclusion was based.

Another method that has been used to deplete ATP in cardiac muscle is 2-deoxyglucose (2-DG) infusion. 2-DG is a glucose analog that, like glucose, is transported into cells and is phosphorylated by hexokinase. Unlike glucose-6-P, 2-DG-P cannot be metabolized further, nor can it exit the cell once phosphorylated. It has been reported that this

results in the breakdown of ATP to AMP to adenosine and finally to inosine, which is released from the cell (78). The validity of this proposed mechanism for the total adenine nucleotide (TAN) depletion may be called into question, though, for several reasons. First, the dephosphorylation of AMP to adenosine by 5'-nucleotidase apparently does not occur to a large extent in non-ischemic conditions (48). Secondly the decline in ATP observed in the ^{31}P-NMR spectra acquired in this study could be accounted for by a corresponding increase in IMP, but this change would have been masked by the large 2-DG-P peak which appears in the same spectral region as IMP. Finally, it is not clear that the chemical assay procedures used in this study were capable of discriminating between inosine and its corresponding nucleotide. In any case, a subsequent study applying this technique to a perfused rat heart preparation established that contractile function was not impaired despite 60-65% depletion of TAN (77).

ATP depletion has also been observed in connection with creatine depletion induced by long-term feeding of creatine analogs to test animals (42, 137, 102). Although these studies uniformly reported unimpaired muscle performance at submaximal workloads, the biochemical and structural adaptations (136, 118, 134) that occur in response to this chronic treatment complicate any interpretations of these results regarding respiratory control.

EXPERIMENTAL DESIGN AND RATIONALE

The experimental portion of the present work consists of two separate studies. The first, as noted in chapter one, was undertaken to

investigate claims of adverse consequences of PNC inhibition on aerobic metabolism in muscle (43). This conclusion was judged suspect for three reasons.

First, the drug AICAr infused in that study is known to interact with numerous other enzyme systems (e.g. 6, 55, 79) in addition to its action as an inhibitor of the final reaction in the purine nucleotide cycle. Secondly, the dose administered was extremely large (2.25 mmol AICAr/100g body weight in a volume of 9 ml/100g, ip). This resulted in an intramuscular AICAr concentration of more than 1 mM (43, 46), far in excess of the amount required for maximal PNC inhibition (129). Finally, previous muscle studies using hadacidin, a drug recognized for the specificity of its action towards PNC inhibition (87), had demonstrated marked PNC blockage with no decrement in either aerobic or anaerobic performance (113, 108).

To examine this question, three separate series of experiments were planned. In the first series, the experiment of Flanagan, et al (43), was replicated with the addition of procedures for monitoring systemic blood pressure, employed on the suspicion of possible systemic effects of the large doses of AICAr. The second phase consisted of another replication of the original experiment, conducted in this case inside an NMR spectrometer in order to monitor the levels of phosphorus metabolites and pH during both stimulation and recovery. The final experimental series utilized isolated cat muscles, perfused with control and AICAr solutions and studied via NMR during stimulation and recovery. The rationale for the use of isolated muscles was that, if the AICAr-associated decline in aerobic performance was indeed due to systemic effects of the drug, then the isolation of the muscle from the remainder

of the system should eliminate this effect on performance. The cat biceps and soleus muscles were chosen for study because of the suitability of their size for the saddle coil probe arrangement and because of the near homogeneity of fiber type, fast and slow respectively, in each muscle.

The second of the two studies in this research plan called for the development of a method to induce and maintain significant ATP depletion in muscles, then for testing of the metabolic effects of this depletion on submaximal twitch and supramaximal tetanic stimulations.

This study also consisted of three phases. In the first phase, anesthetized rats were infused with hadacidin and the right gastrocnemius muscle placed over a surface coil in an NMR probe. The distal end of the limb was then secured to a force transducer to measure isometric force production. The muscle was then electrically stimulated for varying periods of time while phosphorus metabolites were monitored via ^{31}P-NMR to arrive at a protocol which would consistently produce and sustain a maximal depletion of ATP.

The intense stimulation and hadacidn-induced PNC inhibition method for ATP depletion was selected because of the absence of confounding effects as noted in the preceding review. The in situ rat gastrocnemius model was chosen because of the existence of a large body of physical, chemical, and NMR data on that model, as well as the suitability of the animal size and muscle size and location for surface coil NMR studies in the available research magnet. The NMR method was incorporated into these studies for the reasons cited earlier, such as the noninvasive nature of the technique and the superior time resolution ^{31}P-NMR offers for both phosphorus metabolite levels and pH. Traditional chemical

methods were also employed in order to provide an ATP concentration reference for conversion of NMR peak areas into concentration units. The assay results also permitted calculation of total creatine content (PCr + Cr) upon which corrections for contraction-induced muscle swelling could be based.

The second and third experimental series used the protocol developed in phase one to induce depletion of ATP, followed by an interval to allow recovery of other metabolites. In the second series, ATP-depleted muscles were subjected to low frequency twitch stimulations of sufficient duration to produce a steady state of force and metabolite levels. In the last series, the final stimulation bout consisted of trains of tetanic contractions of the same intensity used to induce the initial ATP depletion.

The details of the experimental aims and methods for each of these two major study areas are provided in the two following chapters. Additional background material is also introduced in the interpretations of the experimental results.

III. UTILITY OF AICAR FOR METABOLIC STUDIES IS DIMINISHED BY SYSTEMIC EFFECTS IN SITU

INTRODUCTION

The purine nucleotide cycle (PNC) can be viewed as a two-component process in which adenosine monophosphate (AMP) is first deaminated to inosine monophosphate (IMP), followed by reamination of IMP to AMP to complete the cycle. As described in the previous chapter, AMP deaminase (AMPDA) catalyzes the single reaction of the first component:

$$AMP + H_2O \Longrightarrow IMP + NH_3.$$

The subsequent regeneration of AMP requires two reactions catalyzed in turn by adenylosuccinate (sAMP) synthetase and sAMP lyase:

$$IMP + aspartate + GTP \Longrightarrow sAMP + GDP + P_i$$

$$sAMP \Longrightarrow AMP + fumarate.$$

Inquiries into the physiological role of the PNC in skeletal muscle energy metabolism have focused primarily on three possible functions reviewed by Lowenstein (95): 1). removal of AMP to maintain the ratio of ATP to ADP and AMP; 2). production of ammonia to accelerate glycolysis by activation of phosphofructokinase; and 3). deamination of aspartate, producing fumarate to replenish the supply of Krebs cycle intermediates, thereby enhancing aerobic energy production. Operation of the first arm of the cycle alone is sufficient to carry out the first two functions. Production of fumarate, however, requires a complete turn of the cycle and coincident regeneration of AMP.

Experiments by Tornheim and Lowenstein in 1972 on rat muscle

extracts demonstrated that the generation of ammonia by deamination of AMP occurred during ATP consumption, while reamination of IMP to AMP occurred when conditions favored ATP resynthesis. This prompted the authors to divide the PNC reactions into phases corresponding to 'energy drain' and 'energy excess'(145). A 1977 study by Goodman and Lowenstein using both in situ and perfused rat hindlimb preparations showed that relatively intense stimulation protocols were necessary to cause a net reduction in muscle ATP levels and trigger the production of IMP. Little evidence was found for the cycling of purine nucleotides during work (52).

Clinical interest in the PNC was sparked a year later by Fishbein's report on the discovery of a muscle disorder in humans characterized by the absence of AMPDA, the first enzyme in the cycle. Some victims of myoadenylate deaminase deficiency (MDD) exhibited slight muscle dysfunction during exercise of sufficient intensity to cause lactate accumulation, although others who were identified as MDD by genetic screening had shown no symptoms at all (36). The author speculated that the basis for this mildly impaired function was the inability of MDD muscle to utilize the PNC to maintain a high ATP to ADP ratio by removal of AMP. A more recent clinical finding links reduced sAMP lyase activity and resultant accumulation of sAICAr with infantile autism syndrome (84).

Several recent studies have utilized blockers of the second component reactions to assess the physiological importance of the anaplerotic role of the PNC in muscle. Studies using hadacidin (N-formyl-hydroxyaminoacetic acid), which inhibits the binding of aspartate to sAMP synthetase, indicated that IMP formation occurred only during

relatively intense stimulation, while reamination to AMP was largely delayed until the recovery phase after a burst of contractions (113). Furthermore, production of IMP was shown to occur primarily in fast-twitch glycolytic muscle fibers rather than in fibers with high oxidative potential (108, 114). These studies suggested a serial mode of operation of the two-component cycle, consistent with proposed PNC functions 1 and 2. In contrast, evidence in support of parallel operation of the PNC components was obtained using the compound 5-amino-4-imidazolecarboxamide riboside (AICAr), which is phosphorylated in vivo by adenosine kinase to AICA ribotide (AICAR), an inhibitor of the sAMP lyase reaction (129). In these studies, intraperitoneal administration of AICAr was associated with a marked impairment of the capability of rat gastrocnemius muscle to maintain twitch force during in situ stimulation at 0.75 Hz (43), a rate known to be within the steady-state aerobic capacity of normal rat hindlimb muscle (68). Assuming that the effects of AICAr are confined to sAMP lyase inhibition in muscle, this result suggests that the second arm of the PNC is required for normal aerobic energy production, i.e., that fumarate production is an important physiological role of the PNC in skeletal muscle.

The present study was designed to clarify the effects of AICAr infusion on rat skeletal muscle. The results suggest that the previously reported effects of AICAr on muscle force were secondary to effects on systemic arterial pressure and that AICAr has no direct effect on muscle energy production during mild stimulation.

METHODS

Three separate series of experiments were performed. In the first series, adult male Sprague-Dawley rats weighing 270 ± 10 g (SE, n=16) were anesthetized with pentobarbital sodium (50 mg/kg, ip) and prepared for in situ stimulation of the gastrocnemius muscle essentially as described by Flanagan, et al (43). Three exceptions were made to their reported procedure: a catheter (PE-50) was inserted into the carotid artery for measurement of arterial blood pressure, muscle temperature was maintained at 37°C with a heat lamp throughout the experiment, and stimulation was begun immediately following the 28 minute ip infusion period to ensure a consistent infusion volume (9 ml/100 g, the average infusion volume per animal in the Flanagan report) of either isotonic saline or 250mM AICAr (Sigma Chemical; total dose, 2.25 mmol/100g).

Reviewing the procedure in brief, an ip catheter (PE-50) was inserted for delivery of AICAr or saline, followed by insertion of the carotid catheter. Prior to infusion the right gastrocnemius muscle was exposed and prepared for stimulation via the tibial nerve. During infusion, the distal end of the muscle was connected to a force transducer, and the length of the muscle was adjusted to give a maximal isometric twitch in response to a supramaximal pulse (8 V, 5 ms). AICAr was infused into 10 rats, two of which died during infusion or stimulation and were therefore excluded from the data analysis. A control group of eight rats received an infusion of isotonic saline. Immediately following the infusion period, the muscle was stimulated for 10 minutes at 0.75 Hz. The gastrocnemius muscles of both the stimulated and nonstimulated legs were then immediately clamp-frozen using metal tongs precooled in liquid nitrogen. The frozen samples were stored at

-80° C until extraction.

The presence of AICAr and AICAR in the muscles of the AICAr-infused animals was verified by high resolution ^{1}H-NMR spectroscopy of extracts of the frozen muscles. The frozen samples were pulverized in a mortar under liquid nitrogen, then extracted in cold alcoholic perchloric acid (96). These extracts were diluted 20:1 with deionized water and treated 4 times each with 5g Chelex-100 (BioRad Laboratories) to remove metal ions (82). The chelated product was brought to a pH of 7.0 ± 0.1 using dilute HCl and NaOH solutions, then lyophilized to dryness. The resulting precipitate was redissolved in 1 ml of deuterium oxide (D_2O or $^{2}H_2O$) and transferred to a 5mm NMR tube. The use of D_2O as a solvent eliminates the extremely large ^{1}H peak due to water ($^{1}H_2O$) that tends to obscure other proton peaks in normal aqueous solutions. Standard solutions were prepared by dissolving AICAr and AICAR (Sigma Chemical) in a volume of water equal to the diluted muscle extracts, then chelating, titrating, lyophilizing and redissolving in D_2O as per the muscle extracts to give a final concentration of 100mM for each compound.

Proton NMR spectra of extracts of 6 nonstimulated muscles (3 each, AICAr- and saline-infused), 4 stimulated muscles (2 each group), and the 2 standard solutions were acquired at 400 Hz in a Bruker AM 400 spectrometer while spinning the samples at 25 Hz. Chemical shifts are referenced to the deuterium hydroxide (DHO) peak, assigned a value of 4.80 ppm. Following data acquisition on muscle extracts from the experimental animals, a small amount of the AICAR standard solution was added and the spiked samples reanalyzed. This procedure was then repeated using the AICAr standard.

The second phase of the study utilized a protocol identical to the first, except that the rats were mounted head-down in a custom-made NMR probe as described previously (102), and muscles were stimulated for only 5-6 min. In brief, catheters (PE-50) for AICAr/saline infusion and for supplemental anesthesia (4:1 saline:pentobarbital sodium (50 mg/ml)) were inserted into the peritoneal cavity of the anesthetized rat, and an intraarterial catheter placed as described above. The right sciatic nerve was exposed via a small incision on the lateral aspect of the hip. The nerve was then ligated and cut and the distal end placed in a bipolar platinum electrode. The nerve and electrode were then reinserted into the tissue pocket, using a small strip of Parafilm laboratory film for insulation from surrounding tissues. Cyanoacrylic glue was used to close the incision and secure the position of the electrode. The rat was then positioned in the NMR probe with the knee secured to a mount via a length of copper wire threaded beneath the patellar ligament. The achilles tendon was tied with copper wire to an isometric force transducer located at the top of the probe and mounted on an adjustable frame. Adjustment of the muscle length to optimize isometric twitch tension placed the belly of the gastrocnemius muscle directly over a 1 cm diameter surface coil. Phosphorus NMR spectra were acquired at 162 MHz in a Bruker AM400 7.3 cm bore magnet during infusion and during and after stimulation. A tube delivering 100% oxygen gas was placed near the animal's head to ensure sufficient oxygen delivery within the enclosed probe while inserted in the magnet bore. Eight rats weighing 262 ± 23 g (SE) were tested, four each receiving AICAr and saline infusions as described above. Carotid pressure was monitored in three of the four animals in each treatment group.

In the third experiment, ^{31}P-NMR spectra and twitch force were recorded from isolated, arterially-perfused cat muscles before and after addition of AICAr to the perfusate. Adult cats (3.5-4.0 kg) were anesthetized with ketamine (33 mg/kg, ip) followed by pentobarbital sodium (30 mg/kg, ip). The biceps brachii (a representative mixed fast-twitch muscle) and soleus (slow-twitch) muscles were isolated with their associated vasculature and the muscles were perfused with a suspension of sheep red blood cells in Krebs-Henseleit solution at 30°C as described previously (107). The isolated muscle with attached platinum electrodes was mounted on a force transducer and its length adjusted to give a maximal isometric twitch in response to a supramaximal pulse (15 V, 1 ms). The suspended muscle was inserted into a 20 mm diameter Helmholtz (saddle) coil in a specially designed NMR probe for acquisition of ^{31}P-NMR spectra at 162 MHz. Spectra were obtained before, during and after 6 minutes of stimulation at 0.2, 0.5 or 1 Hz during infusion of control perfusate. AICAr was then added to the perfusate (2.5 mM net AICAr concentration), and after 30 minutes the same stimulation was repeated. One soleus and one biceps muscle were studied, each from a separate animal. It is important to note that each muscle serves as its own control in these experiments.

Statistical comparisons between groups were by Student's t-test at the $p<0.05$ level. Results are expressed as means ± SE, unless noted otherwise in the text.

RESULTS

In Situ Rat Muscle.

Excluding the two AICAr-infused rats that died before the experiment could be completed, there was no significant difference in

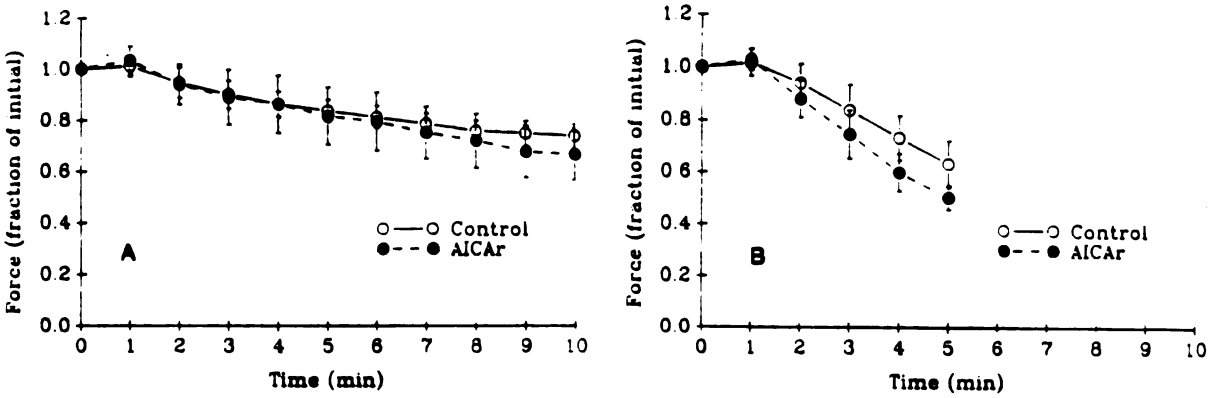

FIGURE 2. Peak twitch force in rat gastrocnemius muscle in situ.

A: 1st experimental series, animals horizontal, n = 8 per group. Initial force was 1280 ± 95 and 1040 ± 160 g in saline- and AICAr-infused, respectively.
B: 2nd series, animals head-down in NMR probe, n = 4 per group. Initial force was 550 ± 55 and 525 ± 55 g, respectively.

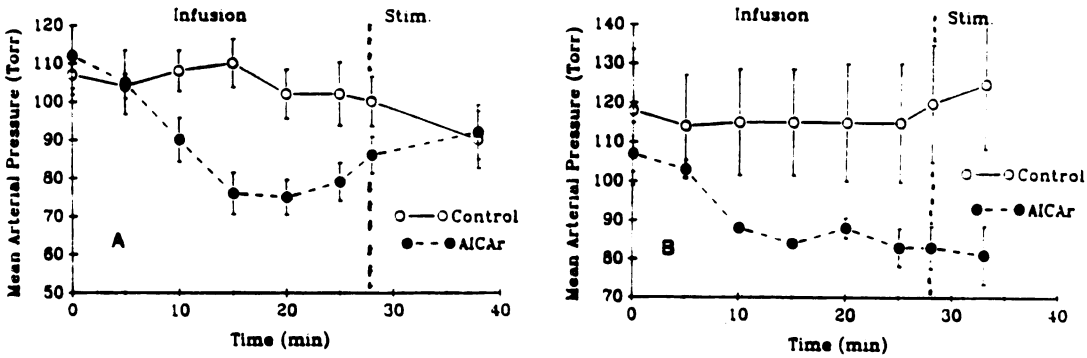

FIGURE 3. Mean arterial pressure in rats during infusion and stimulation.

A: 1st experimental series, animals horizontal, n = 8 per group.
B: 2nd series, animals head-down in NMR probe, n = 3 per group.

fraction of initial twitch force at any time during stimulation in AICAr vs. saline-infused animals (Figure 2A). However, a marked difference between groups was noted in arterial pressure patterns (Figure 3A). Mean arterial pressure in the AICAr-infused rats averaged nearly 20 Torr below those of the control group by 10 minutes of infusion and remained significantly different from control throughout the remainder of the infusion period. Again excepting the animals who died during the experiment, this difference in pressure tended to resolve towards the end of the infusion period, and was totally resolved by the end of stimulation. In the AICAr animal that died (i.e. zero arterial pulse pressure) near the onset of stimulation, twitch force declined to 13% of initial by 5 minutes of stimulation, and to 2% by 10 min.

Proton spectra (Figure 4) of muscle extracts of the AICAr-infused animals showed two series of peaks (7.57,7.54,7.52 ppm, and 5.74,5.72,5.70 ppm) closely corresponding to a single peak and a doublet, respectively, in the AICAR (7.58, 5.71/5.70) and AICAr (7.53, 5.71/5.70) standards. These peaks were present in three of three nonstimulated and two of two stimulated muscles of AICAr-infused animals and were not evident in any of the extracts from control animals. Identification of these peaks as AICAR and AICAr was confirmed by spiking the extracts with standard compounds. In the downfield series, AICAR addition caused a selective increase in the amplitude of the 7.57 ppm peak, whereas AICAr added selectively to the 7.54 and 7.52 ppm peaks, but not to the 7.57 ppm peak. Addition of either compound caused an increase in amplitude of all 3 peaks in the upfield series. The splitting of AICAr and AICAR resonances when added to the muscle extracts presumably reflects conformational changes in these molecules

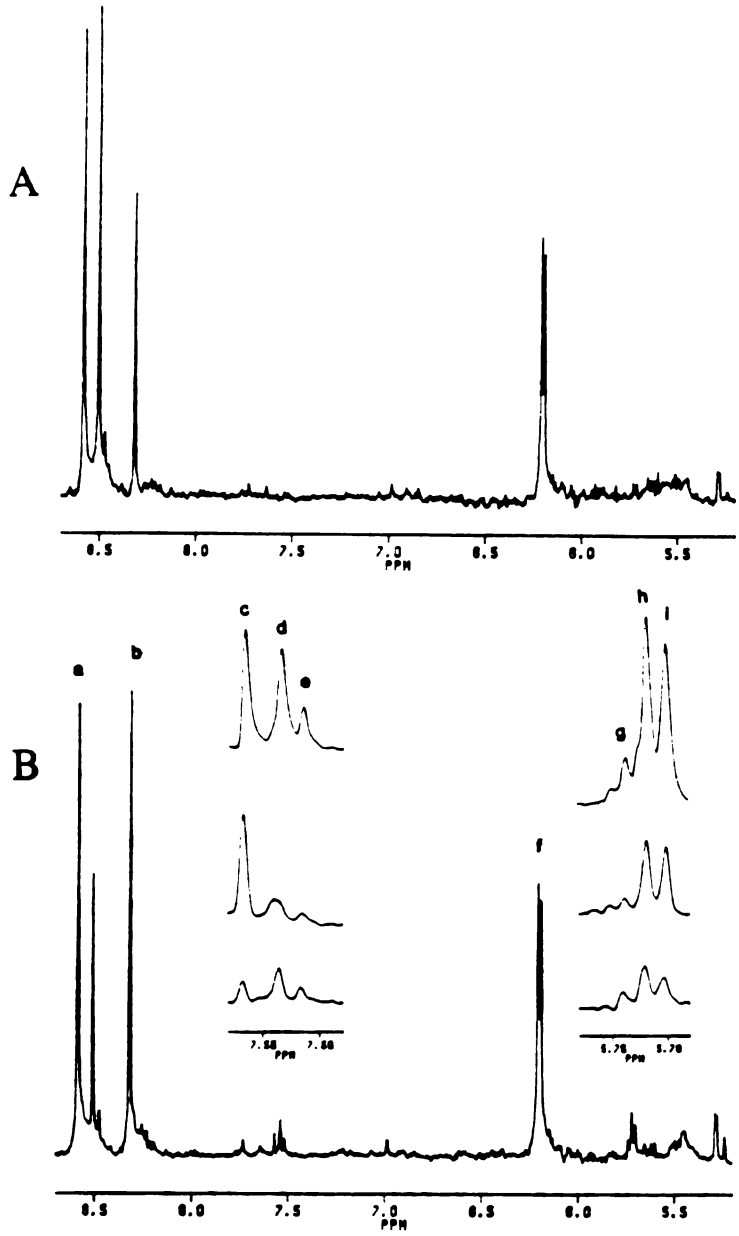

FIGURE 4. ^{1}H-NMR spectra from extracts of rat muscles.

Spectra are from perchlorate extracts of nonstimulated gastrocnemius muscles from saline infused (*A*) and AICAr-infused (*B*) rats. Peak assignments: *a*, *b*, and *f*, adenine nucleotides; *c*, AICAR; *d* and *e*, AICAr; *g*, *h*, and *i*, both AICAR and AICAr. *Inset*: Change in expanded regions of spectra before (bottom) and after sequential addition of AICAR (middle) and AICAr (top). (4000 scans, 1 second interval, sweep width 4 kHz, 16k data.)

due to interactions with other substances in the extracts. series.

The single peaks at 8.59 and 8.31 ppm and the doublet centered at 6.20 ppm have been identified as corresponding to proton resonances from adenine nucleotides (34). If an average total adenine nucleotide content of 8 mM is assumed (107), comparison of the integrals of the adenine nucleotide peaks with those of the AICAR peak at 7.57 ppm yielded an estimated AICAR concentration of 0.5 ± 0.2 mM (n=3) in the nonstimulated muscles of AICAr-infused animals. This estimate is in good agreement with the value of 0.554 ± 0.058 mM measured by high performance liquid chromatography (HPLC) in the Flanagan study (43). The concentration of AICAr averaged roughly twice that of AICAR, as indicated by the greater size of the 7.54 ppm peak (Figure 4). Resonances from lactic acid (1.36 ppm) and creatine-phosphocreatine (3.08 ppm) were also easily resolved in proton spectra of the same extracts. Assuming a total creatine content of 41 mM (83), lactate concentration in the nonstimulated muscles of saline-infused animals was 4.5 ± 0.90 mM (n=3), vs. 9.3 ± 2.01 mM (n=3) for the AICAr group.

<u>In Situ Rat Muscle in NMR Probe.</u>

In AICAr-infused rats mounted head-down in the NMR probe, mean carotid pressure declined from 107±7 to 83±5 Torr (n=3) in AICAr infused rats after 28 minutes of infusion, but was stable (118±15, n=3) in saline-infused animals (Figure 3B). In contrast to the first experiment, this pressure difference persisted during the stimulation. Furthermore, by the end of the infusion period but before stimulation, ^{31}P-NMR spectra (Figure 5) showed a significant increase in the ratio P_i:PCr in muscles of AICAr-treated (0.19±0.04, n=4) animals compared with controls (0.11±0.03). Despite these differences, there was no

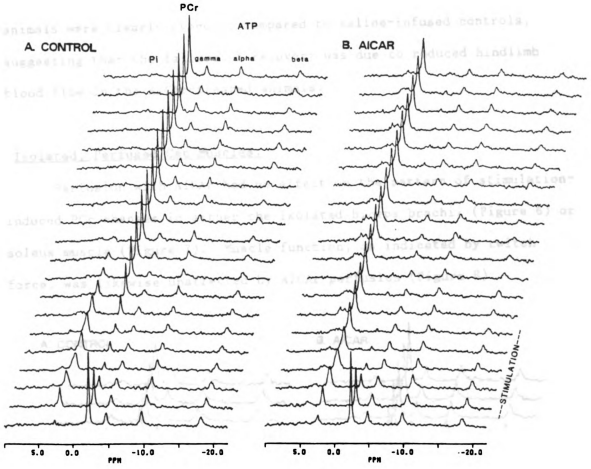

FIGURE 5. ^{31}P-NMR spectra from rat gastrocnemius muscle in situ.

Spectra acquired before (bottom spectrum), during (next 5 spectra) and after 0.75 Hz stimulation of a saline infused (*A*) and an AICAr-infused (*B*) animal. Each spectrum is average over one minute (20 scans, 3 second interval, sweep width 7 kHz, 2k data).

significant difference in twitch force between the two groups after 5 minutes of stimulation at 0.75 Hz (Figure 2B). Phosphocreatine resynthesis after stimulation was dramatically impaired in the muscles of the AICAr-infused rats compared to controls (Figure 5). However, upon removal from the NMR probe, the hindlimbs of the AICAr-treated animals were clearly cyanotic compared to saline-infused controls, suggesting that the failure of recovery was due to reduced hindlimb blood flow in the AICAr-treated animals.

Isolated, Perfused Cat Muscles.

Perfusion with AICAr had no effect on the pattern of stimulation-induced PCr changes in either the isolated biceps brachii (Figure 6) or soleus muscle (Figure 7). Muscle function, as indicated by twitch force, was likewise unaffected by AICAr perfusion (Figure 8).

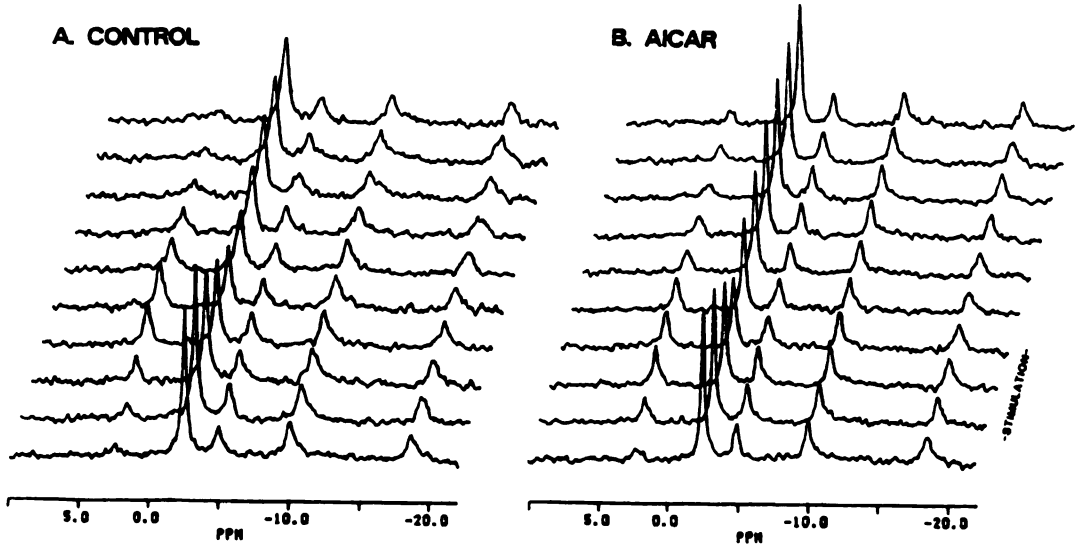

FIGURE 6. ^{31}P-NMR spectra from isolated cat biceps muscle.

Spectra acquired before (bottom spectrum), during (next 3 spectra) and after stimulation at 0.2 Hz. (*A*) is before and (*B*) is 30 minutes after the addition of 2.5 mM AICAr to the perfusate. Each spectrum is an average over two minutes (40 scans, 3 second interval, sweep width 7 kHz, 2k data).

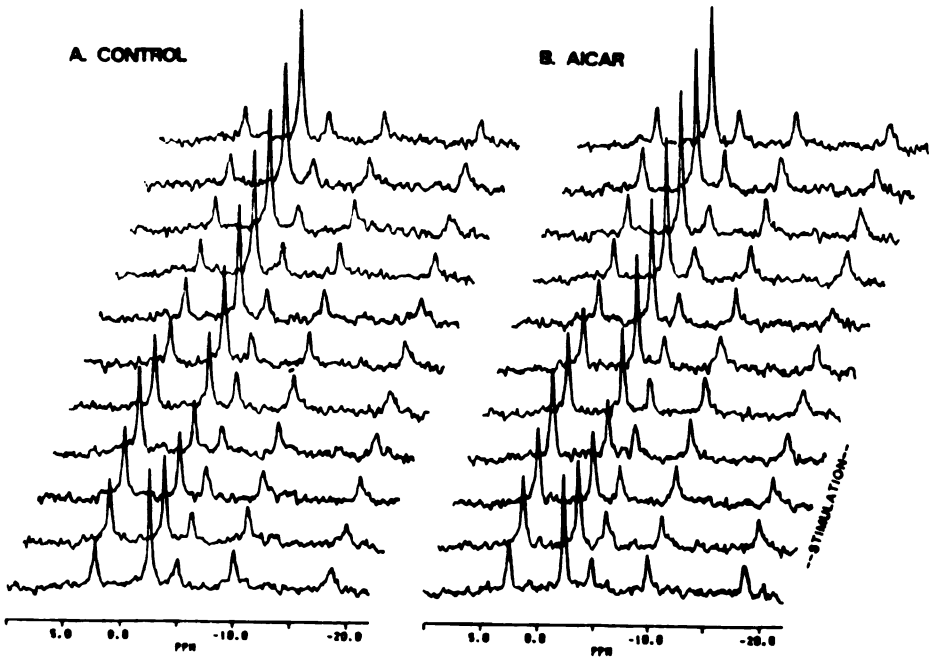

FIGURE 7. ^{31}P-NMR spectra from isolated cat soleus muscle.

Spectra acquired before (bottom spectrum), during (next 3 spectra) and after stimulation at 1 Hz. (*A*) is before and (*B*) is 30 minutes after the addition of 2.5 mM AICAr to the perfusate. Each spectrum is an average over two minutes (40 scans, 3 second interval, sweep width 7 kHz, 2k data).

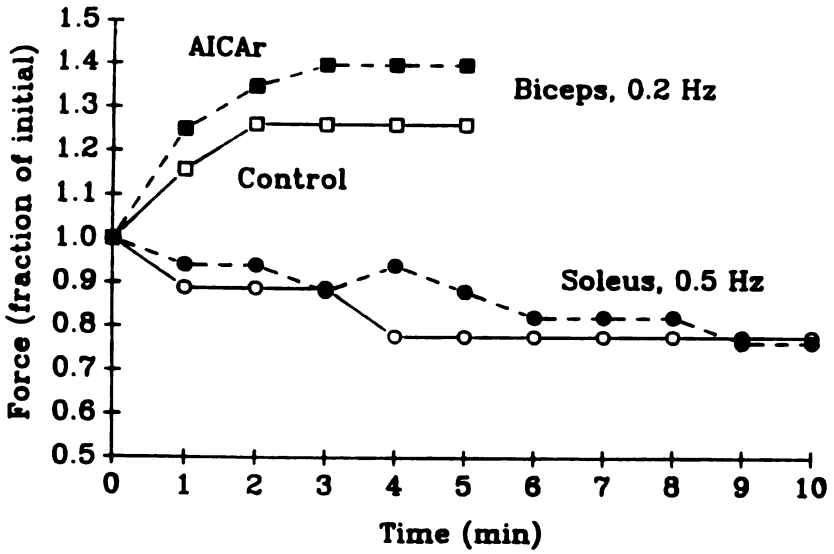

FIGURE 8. Peak twitch force in isolated cat muscles.

Open symbols are before and filled symbols are 30 minutes after the addition of 2.5 mM AICAr to the perfusate.

DISCUSSION

In contrast to previous reports (43, 141), the results of the first series of experiments indicate that AICAr infusion does not impair muscle function during short term, mild intensity, serial contractions. These results, using a stimulus intensity of 0.75 Hz for 5-10 minutes, are consistent with previous results obtained using hadacidin in the same animal model at 1, 3 and 5 Hz (113). However, our results using AICAr contrast markedly with those reported by Flanagan, et al (43), in which AICAr infusion was associated with a rapid decline of twitch force to 25-30% of initial by 5 minutes of stimulation vs. a decline to 90% of initial in control animals. Twitch force in both AICAr and control groups in our study showed a similar drop to ≈85% of initial, despite the significant drop in blood pressure in the AICAr group as noted earlier. Inasmuch as the AICAR content of AICAr-infused muscles was similar in the two studies, this discrepancy cannot be attributed to differences in the uptake or metabolism of AICAr. We propose that differences in hemodynamic state of the animals may explain the muscle performance variation between studies.

In our experiment, arterial pressures fell below 80 Torr for at least 10 minutes during infusion in 9 of 10 experimental animals and in only 1 of 8 controls. If the cardiovascular state of the animal is already compromised prior to stimulation, as is apparently the case in the Flanagan experiment, the additional stress of an AICAr-induced suppression of blood pressure might be expected to accelerate muscle fatigue in the experimental group relative to controls. In the absence of this pre-existing ischemic condition, the muscle may be capable of performing brief, mild work using intramuscular fuel supplies even if

AICAr-induced systemic effects were to limit the muscle's access to circulating substrates.

The second experimental series in this study, in which rats were mounted head-down during infusion and stimulation, provides insight into the effect of hemodynamic alterations on in situ muscle metabolism and performance. Twitch force decreased faster in both AICAr- and saline-infused groups in this series compared to the first. The vertical position would be expected to decrease arterial pressure at the hindlimb by only about 15 Torr compared to the carotid pressure. However, an additional factor limiting perfusion of the hindlimbs in these experiments may have been the presence of a large volume of infused fluid. In the vertical position, this fluid would rest on the diaphragm, thus restricting venous return. Under these circumstances, the drop in arterial pressure associated with AICAr infusion might have had a dramatic effect on hindlimb flow, and hence on recovery metabolism. The observation of cyanosis in hindlimbs of AICAr-infused rats is consistent with this interpretation.

Despite the clearly depressed rate of PCr recovery and the visual signs of ischemia associated with AICAr infusion in the second series of experiments, there was still only a slight effect of AICAr infusion on twitch force compared to controls. This is not really surprising, because the intracellular stores of PCr and glycogen in rat gastrocnemius (a predominantly fast-twitch muscle with high glycolytic capacity [5]) are normally sufficient to maintain some force development for several minutes during stimulation at 0.75 Hz, assuming an energy cost of around 0.2 umol ATP/twitch (68). This is illustrated by the results from the muscle of the animal that died (zero arterial pulse

pressure) just prior to stimulation, and yet still developed 13% of initial twitch force after 5 minutes of stimulation, and 2% after 10 min. Failure to recover from this depletion of endogenous fuel only in the AICAr group is consistent with a severe restriction of peripheral blood flow blocking access to the blood-borne substrates needed to replenish depleted muscle fuel supplies.

It is well known that muscle twitch force is sensitive to variations in arterial pressure during in situ stimulation experiments (65). In the present study, AICAr infusion resulted in a significant decrease in mean arterial pressure compared to saline infusion. It follows from the above analysis of energy cost that, if the decrease in twitch force to 18 ± 13% after 10 minutes stimulation in muscles of AICAr infused animals reported by Flanagan, et al, was due to decreased limb perfusion, then the effect on perfusion must have been more prolonged or more severe in that study compared to this one. The noted alteration of method in this study, whereby infusion was stopped prior to stimulation, suggests the possibility of a more prolonged depression of pressure in Flanagan's study, in which infusion was continued throughout the stimulation period. In the first experimental series in this study, the pressure difference between groups resolved during the stimulation period, and this recovery of pressure in the AICAr group coincided with the termination of infusion.

Several other features of Flanagan's data suggest that the hemodynamic state of the animals may have been more severely compromised than in this study. First, PCr content was decreased by 15% in unstimulated muscles of AICAr-infused compared to their control animals. Second, the lactate concentrations reported by Flanagan, et al, were

over 10 mM in unstimulated muscles of both saline- and AICAr-infused groups. This is somewhat more than in this study (4.5 and 9.3 mM, respectively) and also more than previously reported following preparation of rat gastrocnemius for similar hindlimb experiments (2-3mM [108]). Taken together, these data suggest that the animals in both groups of Flanagan's study were hemodynamically compromised prior to the start of stimulation. Under these conditions, the additional suppression of arterial blood pressure associated with AICAr infusion might well have a profound effect on twitch force.

Operation of the PNC.

Two interpretations of these results are possible. Since both AICAr and hadacidin block the reamination arm of the cycle, it could be concluded that the first component alone is required for maintaining muscle function. Alternatively, it may be argued that none of the PNC reactions may even be occurring to any significant extent, given the low intensity of the workload. The likelihood of this second possibility is supported by a number of observations. A study by Meyer and Terjung showed that 30 minutes of treadmill running at 80% of maximal oxygen consumption produced no evidence of AMP deamination or ammonia production (113), indicating that AMPDA is not active under these conditions. It has also been demonstrated that a stimulation rate of 0.75 Hz is within the steady state work capacity of rat gastrocnemius muscle (83, 143). Indeed, no decline in ATP levels was observable in this muscle during stimulation at rates up to 1 Hz. Hence several factors necessary for the activation of AMPDA are lacking under conditions of stimulation at 0.75 Hz, namely lactate accumulation and

resulting acidosis, as well as a drop in ATP levels indicating a declining energy charge in the muscle cell.

It has been shown that the major regulators of AMPDA in vivo are H^+ and free ADP and AMP (activators) and P_i (inhibitor), and that the resting levels of these compounds probably effectively inhibit AMPDA (149). This enzyme exhibits a pH optimum of 6.5 under in vivo conditions, and has been shown to be essentially inactive until muscle lactate accumulates to a concentration exceeding 20 mM (27). Under strenuous exercise conditions, rising ADP levels serve to relieve the inhibition of AMPDA by P_i, and this effect is accentuated by lowered pH (149).

The ^{31}-P NMR results of the second series of experiments (Figure 5) show that this stimulation regimen produced an increase in P_i without reducing ATP levels or greatly altering pH. Under these conditions, P_i inhibition of AMPDA should increase (K_i of P_i at pH 7.0 = 1.3 mM [149]) while the concentrations of activators show little change. Therefore, it seems unlikely that the PNC is operative at all under these conditions. If this is the case, inhibitors of the second phase of the cycle would not be expected to affect muscle function. The failure of AICAr infusion to affect either contractile response or phosphagen profiles in the perfused muscle experiments supports this conclusion.

If AMPDA is not significantly activated during mild contraction sequences, then inhibition of the reamination reactions, and therefore of the anaplerotic potential of the PNC, would have little physiological impact. Furthermore, there is little evidence that the anaplerotic role of the cycle is physiologically important even under conditions where significant cycling can be demonstrated. For example, Flanagan, et al,

did find significant increases in both IMP and sAMP in muscles of AICAr compared to saline-infused rats (43), suggesting that some increase in PNC turnover had occurred. However, there were no statistically significant differences in measured Krebs cycle intermediates (malate, citrate) between the two groups after stimulation. Thus, even if the drastically reduced twitch force in that study were due to PNC inhibition, it would be difficult to attribute this to a limitation of Krebs intermediates.

Systemic Effects of AICAr.

The decline in arterial blood pressure associated with AICAr infusion in our study even before any muscle stimulation demonstrates that AICAr's pharmacological effects are not confined to skeletal muscle cells. The impairment of PCr resynthesis following stimulation is also consistent with a systemic cardiovascular response to AICAr. The failure of AICAr to have any effect on PCr recovery in the perfused cat muscles also argues that the effect in rats was not primarily on muscle.

The issue of systemic effects of AICAr has been explored in several previous studies. Sabina, et al, concluded from a study involving atrial infusion of 100 mM AICAr into dogs that the compound was hemodynamically inert, although the supporting data was not shown (128). In contrast, a study of recovery of canine myocardium from ischemia by occlusion noted that reperfusion with 9mM AICAr caused a "marked impairment of regional [myocardial] function which progressed with time" compared to saline-infused controls (66). Intravenous injection of derivatives of the base AICA have been shown to cause behavioral sedation in mice as well as dilation of peripheral blood vessels and

suppression of blood pressure in cats and rabbits (55).

Identification of potential pharmacologic targets for AICAr may help clarify the basis for this complex array of reported effects. In addition to the inhibition of sAMP lyase by the monophosphorylated form of AICAr, it has long been known that AICAr itself is a potent competitive inhibitor of E. Coli adenosine deaminase, which has a K_i for AICAr of 9 μM vs. a K_m of 50 μM for adenosine (79). A more recent report demonstrated that AICAr is a competitive inhibitor of bovine adenosine deaminase, with a K_i of 0.362 mM vs. a K_m of 0.017 mM for adenosine (6). The tissue accumulation of AICAr in excess of 1 mM in our experiment would certainly imply that a marked impairment of adenosine deamination could be occurring under these conditions. Of related interest is a proposal by Baggot, et al, that inhibition of adenosine deaminase due to a buildup of AICAr may be the basis of methotrexate cytotoxicity (6).

Baggot, et al (6), also reported that AICAR inhibits AMPDA, the first enzyme of the PNC, further diminishing the utility of AICAr in separating the functions of the first and second components of the PNC. The AICAR accumulations reported here and by Flanagan (43) average more than half the reported K_i value of 1.01 mM (6), enough to cause some interference with the AMPDA reaction. Indeed, Flanagan's data support such a thesis: the IMP/AMP ratio after tetanic contractions was nearly 40% lower in AICAr-infused animals than in controls, suggesting that deamination of AMP to IMP was somewhat impaired in the experimental group (43). These data contrast with IMP/AMP ratios that were 250% higher in intensely stimulated muscle of hadacidin-treated rats compared to controls (113).

The biochemical basis for these interactions of AICA-based compounds with adenine-based enzyme systems lies in the structural similarity between AICAr and the purine nucleosides (Figure 9). The presence of a rotatable bond between C5 and C6 of the base AICA allows the molecule to assume conformations analogous to either adenine (A form) or guanine (G form) (130). In the A form, the structures of AICAr and AICAR mimic those of adenosine and AMP respectively, creating the possibility for interaction with numerous enzymes such as those documented above.

Both AICAR and succinyl-AICAR are precursors of IMP in the de novo pathway for purine nucleotide synthesis (57). AICAR is converted to IMP in the two final steps of this pathway by addition of a formyl group and elimination of water to create the closed purine ring system. The conversion of AICAr to AICAR, a condition necessary for inhibition of sAMP lyase, could therefore also be followed by the subsequent conversion of AICAR to IMP via the de novo synthesis pathway. IMP produced by this route could conceivably confound the results of AICAr studies regarding the PNC-related changes in IMP levels. Furthermore, even if a decrease in fumarate production due to reduced conversion of sAMP to AMP actually did occur, this effect might be masked by the generation of fumarate by the action of sAMP lyase on sAICAR (57), which has been shown to accumulate in AICAr-infused rats (43).

The interaction of AICAr with adenosine deaminase is of particular interest in view of the hemodynamic effects of AICAr demonstrated in this study. The tissue accumulation of AICAr in these experiments to nearly three times the K_i for adenosine deaminase inhibition could cause marked impairment of adenosine degadation. The resulting buildup of

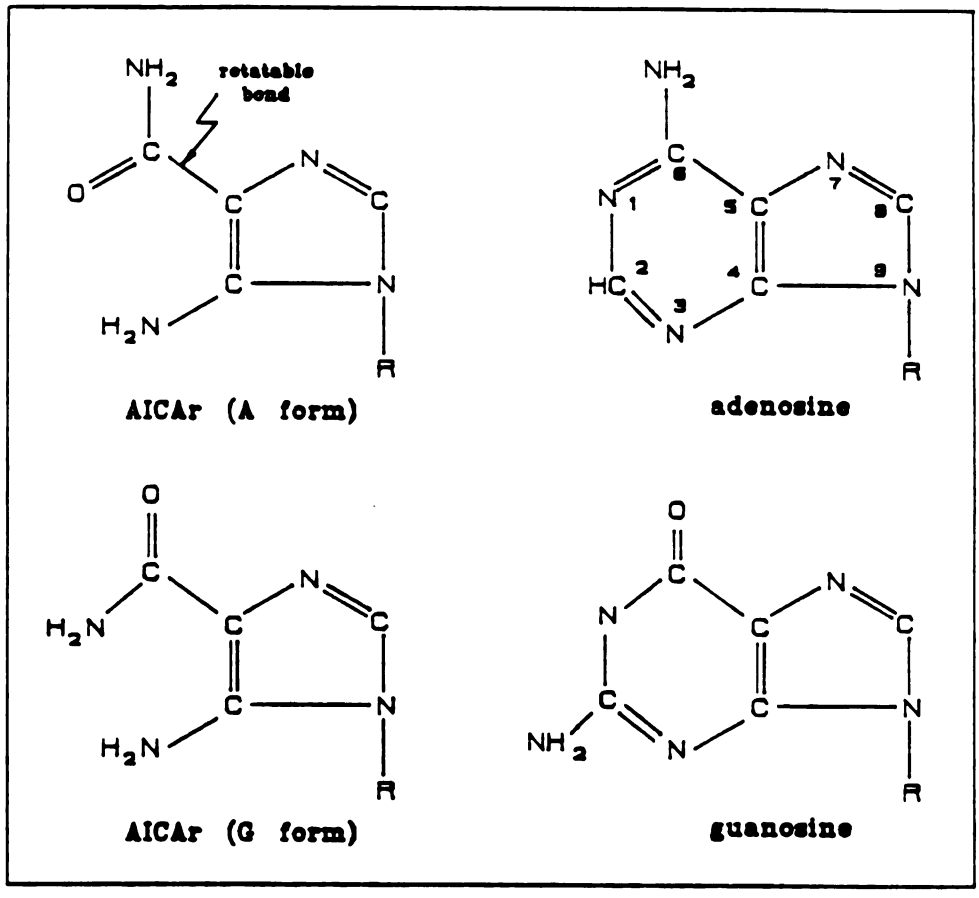

FIGURE 9. Conformers of AICAr compared to adenosine and guanosine.

this potent vasodilator could reduce cardiovascular tone, causing a pressure drop as observed in this study. It has been shown that contractions performed under ischemic conditions are likely to activate 5'-nucleotidase, causing degradation of AMP to adenosine (150). This could result in a positive feedback loop in AICAr-infused animals, producing a profound pressure drop. This effect would be exacerbated by preexisting ischemia, perhaps explaining the reduced force production by AICAr-treated muscles relative to controls as reported in the Flanagan study (43).

In summary, our results indicate that infusion of AICAr into rats has a significant effect on systemic arterial pressure, perhaps by inhibiting breakdown of the vasodilator metabolite adenosine, but has no effect on muscle force generation during moderate stimulation. The results do not support the hypothesis that the PNC performs an important anaplerotic role in muscle aerobic metabolism. Furthermore, the systemic effects of AICAr, as well as the documented interaction of AICAr and its metabolites with numerous other enzyme systems, call for caution in interpreting results of studies using this compound and advise against its use in future studies of the purine nucleotide cycle.

IV. ACUTE ATP DEPLETION DECREASES PHOSPHOCREATINE USE AND ACID ACCUMULATION DURING MUSCULAR CONTRACTION

INTRODUCTION

The role of ATP as the physiological energy currency is well established. ATP levels are particularly high in skeletal muscle, in which the rate of ATP turnover can increase by three orders of magnitude during a transition from rest to work (80). Furthermore, the ATP content of different muscle types is generally correlated with actomyosin ATPase activity and shortening velocity. For example, the ATP content of mammalian fast twitch muscles (6-8 μmol/g) is roughly twice that in slow twitch muscles (3-5 μmol/g) (114). During all but the most extreme contractile conditions, ATP levels in both muscle types are maintained by oxidative and glycolytic metabolism, as well as by the creatine kinase system (112). Even in the extreme case of prolonged intermittent tetanic contraction, ATP declines by only about half in fast twitch muscle, and even less in slow twitch muscle (114).

The question therefore arises: is there a functional requirement for the high level of ATP in fast twitch muscle? Based on studies of skinned fibers (17, 26), as well as on measurements of the kinetics of isolated myosin (147), it does not appear likely that increases in ATP above 1-2 mM could affect the contractile apparatus. Similarly, assuming that whole muscle measurements accurately reflect cytosolic metabolite concentrations, the ATP concentration in muscle is far greater than the K_m for ATP of most other enzyme systems. Chronic depletion of PCr and ATP in skeletal muscle by administration of creatine analogs (102, 137), and acute depletion of PCr and ATP in heart muscle by 2-deoxyglucose

perfusion (77) have only marginal effects on force. Although ATP depletion is correlated with decreased force during repetitive or ischemic stimulation of intact muscles (143), such stimulation has many other effects, including acidification and increased phosphate content, which are more likely to effect force. Thus, the functional significance of the high ATP content in fast twitch muscle is obscure.

In this study of a mixed fast twitch muscle, acute and selective depletion of ATP and total adenine nucleotide content was accomplished by inhibiting their resynthesis from IMP during recovery after prolonged intermittent tetanic contraction. The rat gastrocnemius muscle used is composed of 95% fast twitch fibers (58% fast glycolytic (fast white) and 37% fast oxidative glycolytic (fast red)) and 5% slow twitch fibers (slow oxidative or slow red) (5).

The general aim of the study was to examine the effect of ATP depletion on muscle metabolism and isometric force generation. Specifically, we wished to test a simple prediction of the theory that muscle respiration is kinetically controlled by cytoplasmic ADP concentration. Assuming equilibrium of the creatine kinase reaction (103, 146), cytoplasmic ADP content is directly proportional to ATP content, as well as to the creatine-to-PCr ratio:

$$(A) \quad [ADP] = [ATP]^* ([Cr]/[PCr]) * (1/[H^+]) * 1/K_{eq}$$

Therefore if ATP is depleted by half, the creatine-to-phosphocreatine ratio must increase 2-fold (or the hydrogen ion concentration decrease by half) to maintain the same cytosolic free ADP concentration. Assuming constant total creatine (83, 102), it follows that if oxidative metabolism is controlled by cytoplasmic [ADP], then PCr should be lower

(or pH higher) in ATP-depleted muscles compared to control muscles respiring at the same rate. Thus, PCr should decrease more in ATP-depleted compared to control muscles during twitch stimulation at rates known to be sustained by aerobic metabolism.

METHODS

Adult male Sprague-Dawley rats (Harlan-Sprague-Dawley, Madison, WI) were housed three per cage in a temperature controlled room (22°C) on a 12 hour light-dark schedule, with water and standard rat chow provided ad libitum. Body weights averaged 294 ± 32 (SD, n=16), 368 ± 32 g (n=10,) and 352 ± 19 (n=8), respectively for the three experimental series described in sequence below.

Surgical Preparation and Stimulation.

Animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and prepared for in situ stimulation of the right gastrocnemius muscle group within a phosphorus NMR probe as described previously ((102), also chapter three). In brief, the right sciatic nerve was dissected free, cut and placed in a bipolar platinum electrode. The right knee of the supine rat was fixed in place and the tendon was attached to a position-adjustable strain gauge force transducer, placing the gastrocnemius muscle directly over a 1 cm diameter surface coil. Body temperature (typically 36-37 °C) was monitored throughout the experiment by a rectal temperature probe, and the NMR probe was ventilated with 100% oxygen while enclosed within the magnet bore. Additional anesthetic was administered in 5 mg doses approximately every 30 minutes via an indwelling ip catheter. Hadacidin (100 mg/kg per dose in a solution of

21 mg/ml) or normal saline (controls; same volume per dose) was delivered through a second ip catheter. The hadacidin used in these experiments was a gift from Merck, Sharp & Dohme Research Laboratories, Rahway, NJ.

Muscle length was adjusted to give a maximal isometric twitch in response to a supramaximal pulse (10-20 V, 2 ms). Fifteen minutes after the initial hadacidin or saline injection, the muscle was subjected to 3 minutes of intermittent tetanic stimulation (100 Hz, 100ms, 1 Hz train rate) while force was monitored via the transducer arrangement as described above. A second dose of hadacidin or saline was then administered, the leg was restretched to optimal length, and a second, identical stimulation bout was given 5 minutes after the end of the first bout. Pilot NMR studies showed that this dual-stimulation bout protocol produced a fairly consistent 50% depletion of ATP, whereas a single stimulation bout gave more variable results and averaged only 30% depletion, a result consistent with previous reports (110).

After the second tetanic stimulation bout, the nerve contact was repositioned distally and the leg was again restretched to produce a maximal twitch. The probe was tuned to 162 MHz and inserted with the animal head downward into a Bruker AM400 spectrometer (7.4 cm bore vertical magnet) for the final stimulation phase. The field homogeneity was shimmed on the muscle water proton signal to a line width of 40-70 Hz and pulse width was chosen to optimize the signal-to-noise ratio as described previously (102). Unsaturated ^{31}P-NMR spectra (162 MHz, 7000 Hz sweep width, 20 scans, 15 s interval, 5 minutes blocks) were then continuously acquired until phosphorus metabolite levels and pH, as indicated respectively by peak areas and chemical shift of the inorganic

phosphate peak, had stabilized. A final dose of hadacidin or saline was administered 30 minutes after the second dose.

Upon recovery to a stable metabolic state (typically 75 minutes after end of the initial stimulation protocol) the muscle was subjected to a final stimulation bout. In one set of experiments, muscles (n=8 each, control and ATP-depleted) were stimulated at 0.75 Hz for 8 minutes. Muscles of a second set of animals (n=5 per group) were subjected to another bout of intermittent tetanic stimulation (100 Hz, 100 ms trains at 1 Hz for 3 minutes). In both cases, spectra were continuously acquired in 30 second blocks (16 scans plus one dummy scan, 1.76 s interval, 7 KHz sweep width, 2K data) to monitor the time course of PCr, ATP, P_i, and pH changes before, during and after stimulation. Finally, an unsaturated spectrum (20 scans, 15 s interval) was acquired. Both control and stimulated muscles were then excised and immediately clamp frozen between aluminum blocks precooled in liquid nitrogen. The frozen samples were stored at -80°C for later extraction and chemical analysis.

A third series of experiments followed the same general procedure as outlined for the above NMR experiments, but with the following exceptions. These experiments were conducted on the bench rather than in the NMR spectrometer. Arterial blood pressure was monitored continuously via a catheter (PE-50) inserted in the right carotid artery. The gastrocnemius muscle groups in both legs were exposed and the tibial nerves tied and cut as described previously ((114), chapter three). The distal tendon of the right muscle of the prone rat was secured to a force transducer, and the exposed muscle was kept moist with normal saline and maintained at 37°C. Four rats per group were subjected to the

same tetanic stimulation and recovery procedure as described above. Force records of single test twitches and tetani (100 Hz, 500 ms duration) were recorded at 250 mm/sec chart speed at intervals throughout the experiment to permit measurement of contraction rise and relaxation times.

NMR Analysis.

Peaks were integrated using a Lorentzian fitting algorithm (109) on the Fourier transform of the summed free induction decay multiplied by an exponential corresponding to 25 Hz line broadening. Relative contents of IMP, inorganic phosphate (P_i) and PCr in resting muscle after the stimulation/recovery protocol were estimated from areas of the corresponding peaks in unsaturated spectra. ATP levels were calculated by averaging the areas of the Γ and β phosphate peaks of ATP. Areas were scaled to μmol/g assuming a total phosphate integral (sum of IMP, P_i, PCr, and the three ATP peaks) of 51 μmol/g (83, 102). For partially saturated spectra acquired during and after the final stimulation, peak areas were scaled relative to areas in the spectra acquired immediately before stimulation, and multiplied by the metabolite content (μmol/g) as determined from the unsaturated spectra. Previous studies have demonstrated no significant change in apparent T_1 nor selective loss of signal for any NMR-observable phosphate metabolites in this muscle using this protocol (106). Intracellular pH was estimated from the observed chemical shift (δ_{obs}) of the inorganic phosphate peak (120) according to the formula

$$(B) \qquad pH = 6.87 + \log[(0.89 - \delta_{obs})/(\delta_{obs} - 3.19)].$$

ADP content was calculated per equation A from PCr, ATP and pH, assuming a constant total creatine content of 41 μmol/g (83, 102), and assuming equilibrium of the creatine kinase reaction (K_{ck}=1.66x10^9 M^{-1} (72)).

Chemical Analysis.

The frozen muscles were pulverized in a mortar under liquid nitrogen, and extracted with cold alcoholic perchloric acid (96). The resulting extracts were stored at -80°C and later assayed in duplicate for ATP, PCr, and Cr using standard enzymatic techniques (114). Metabolite recovery exceeded 90% for all assay systems. In order to correct for variations in tissue water content between stimulated and nonstimulated muscles (114), results were normalized to a constant total creatine content (41 μmol/g, (83, 102)). Enzymes and standards were obtained from Sigma Chemical, St. Louis, MO.

Statistical Analysis.

Data are reported as means ± SE unless noted otherwise in the text. Statistical comparisons were by Student's t-test or analysis of variance at the $p<0.05$ level of significance.

RESULTS

Effect of the ATP Depletion Regimen.

Phosphorus NMR spectra typical of control and hadacidin-treated (ATP-depleted) muscle following the stimulation/recovery protocol described above are shown in Figure 10. The two initial bouts of tetanic stimulation resulted in loss of roughly half of initial ATP stores coincident with a stoichiometric rise in IMP in both treated and control muscles, as expected in response to this intense metabolic stress

(114, 148). ATP recovered gradually toward initial levels in control muscles but not in the hadacidin group, again in agreement with previous reports (113). Analysis of the NMR spectra acquired after 75 minutes of recovery (Tables 2 and 3) showed that hadacidin-treated muscles had 32% less ATP than controls. Both IMP and inorganic phosphate were increased in hadacidin-treated muscles compared to controls.

There was also a slight but significant decrease in intracellular pH (Table 3) in hadacidin-treated compared to control muscles. Calculated free ADP in the hadacidin-treated muscles was half that in controls, while phosphorylation potential ($\ln[ATP]/([ADP]*[P_i])$) was similar in the two groups. The pH and relative metabolite contents in control muscles after the recovery period closely match those observed in nonstimulated muscles in previous NMR studies (83, 102).

These NMR results were confirmed by chemical analysis of muscles frozen after the final twitch stimulation (Table 4). ATP in hadacidin-treated muscles was decreased by over 50% compared to nonstimulated muscles, and by 45% compared to identically stimulated control muscles. Furthermore, the chemical analysis revealed a significant increase in PCr content in ATP-depleted compared to control muscles. This trend was also evident in the NMR analysis (Table 3) but there it did not achieve statistical significance. A similar increase in PCr was previously observed in extracts of hadacidin-treated compared to control rat white vastus lateralis muscles subjected to a stimulation-recovery regimen similar to that used in this study (Meyer, Dudley, and Terjung; unpublished results).

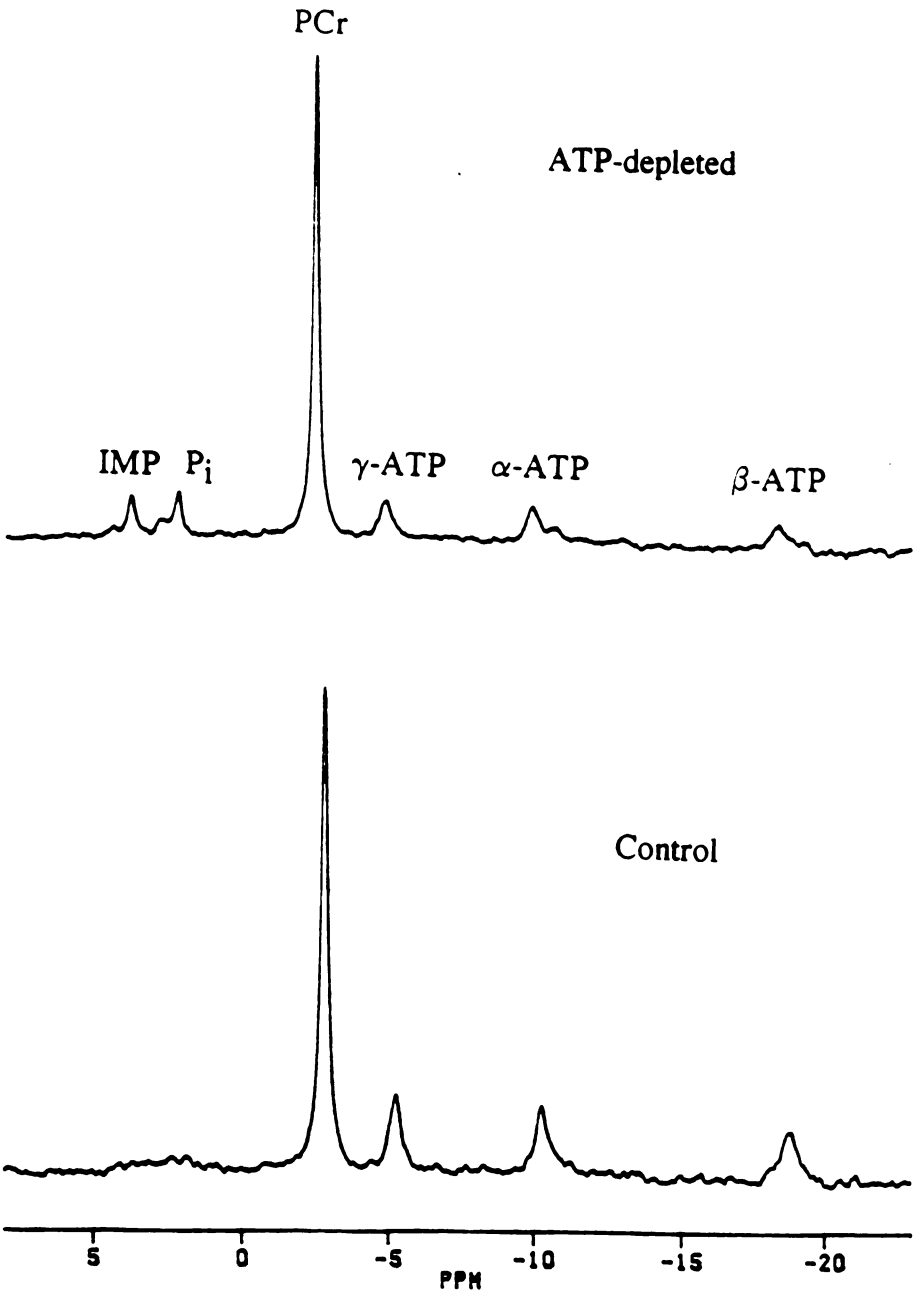

FIGURE 10. ^{31}P-NMR spectra from rat gastrocnemius muscles after recovery from rigorous tetanic stimulation.

Bottom spectrum is from control and top is from hadacidin-treated muscle after recovery from tetanic stimulation as described in text. Each spectrum is average over 10 minutes (40 scans, 15 s interval, 7 KHz sweep width, 2K data).

TABLE 2. Relative peak areas in ^{31}P-NMR spectra of rat gastrocnemius muscle after 75 minutes recovery from tetanic stimulation protocol.

	Control	Hadacidin-treated
IMP	1.0±0.4	6.1±0.7 *
P_i	4.1±0.9	7.9±0.4 *
PCr	54.1±1.1	58.0±2.0
Γ-ATP	12.8±0.7	9.4±0.5 *
α-ATP	12.2±0.6	7.8±0.8 *
β-ATP	15.8±0.8	10.7±1.9 *

(areas are expressed as fraction of total integral, mean ± SE, n=12)

* Significantly different from control, $p<0.05$.

TABLE 3. Metabolite contents estimated from ^{31}P-NMR spectra of rat gastrocnemius muscle after recovery from tetanic stimulation protocol.

	Control	Hadacidin-treated
ATP (μmol/g)	6.93±0.19	4.76±0.35 *
PCr (μmol/g)	27.58±0.54	29.57±1.01
IMP (μmol/g)	0.51±0.18	3.11±0.33 *
P_i (μmol/g)	2.11±0.44	4.05±0.65 *
pH	7.05±0.02	6.95±0.02 *
ADP (nmol/g)	22.3±1.8	11.1±2.2 *
$\ln(ATP/(ADP*P_i))$	11.87±0.16	11.84±0.23

Metabolite contents calculated from relative peak areas (Table 2) assuming total phosphate integral ($IMP+P_i+PCr+ATP$) equals 51 μmol/g (83, 102). ATP is mean of α, β, and Γ peaks. ADP calculated from equation A in text. All values are mean ± SE, n=12.

* Significantly different from control, $p<0.05$.

TABLE 4. ATP and PCr contents from analysis of extracts of rat gastrocnemius muscles frozen after recovery from final twitch stimulation protocol.

	ATP (μmol/g)	PCr (μmol/g)
Non-stimulated muscle		
Control	7.59±0.59	26.89±0.65
Hadacidin-treated	7.76±0.76	26.90±0.93
Stimulated muscle		
Control	6.23±0.54	26.90±0.94
Hadacidin-treated	3.42±0.54 *+	31.57±0.95 *+

(All values are mean±SE, n=8.)

* Significantly different from non-stimulated muscle of same group.
\+ Significantly different from control, $p<0.05$.

There was no effect of hadacidin treatment on ATP or PCr content of nonstimulated muscles (Table 4). Pilot NMR studies also showed no effect of hadacidin injection on phosphorus metabolite levels or pH in muscles not subjected to any prior stimulation. These results confirm earlier studies (113, 108) which concluded that acute inhibition of IMP reamination has no effect on metabolism of muscle at rest or during submaximal stimulation. Hadacidin treatment also had no effect on systemic arterial blood pressure at any time during the protocol. Initial mean pressures prior to any injection or stimulation were 106 ± 7 and 100 ± 5 Torr in the control and experimental groups respectively. By the end of the protocol mean pressures were 100 ± 2 vs. 99 ± 8, respectively. These pressures are within the normal range reported for this preparation (113, 46).

Figure 11 shows the peak force during the two initial tetanic bouts of the first experimental series. Peak force did not differ between groups at any time during either of these bouts in any of the three sets of experiments. However there was a significant change in the pattern of force development during the second bout compared to the first in both groups. During the first series of tetani, there was an initial rapid decrease in force, followed by a more gradual decline. This pattern has been documented in many previous studies (e.g. 148, 114). During the second series, tension initially increased with successive tetani, peaking at 8-10 seconds, before declining to a final

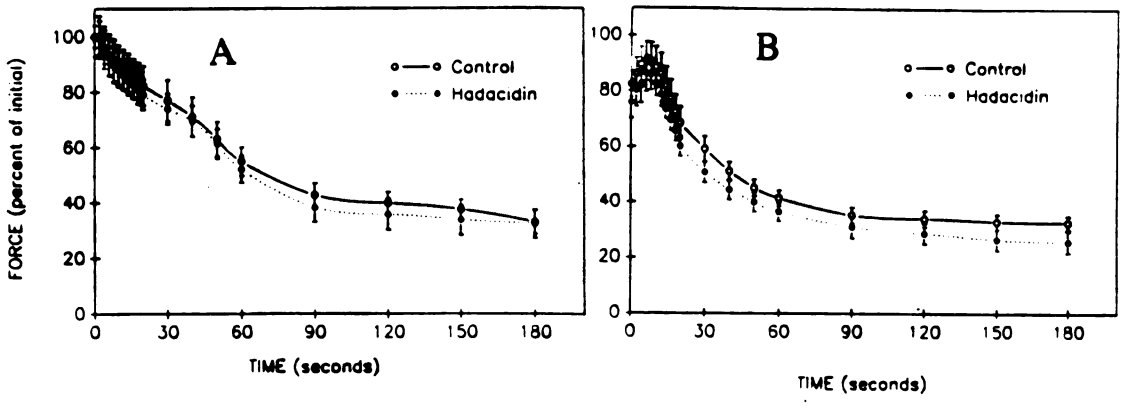

FIGURE 11. Peak isometric force in rat gastrocnemius muscles during successive bouts of tetanic stimulation.

A: First bout of intermittent tetanic stimulation as described in text, commencing 15 minutes after saline or hadacidin injection.
B: Second bout, commencing 5 minutes after end of bout 1.
Points are means ± SE, n = 8 muscles. Initial force (g/g body weight at t=0 of bout 1) was 7.98 ± 0.57 (controls) and 8.55 ± 0.34 (treated).

level similar to that of the first bout. Inasmuch as the altered profile occurred in both control and experimental groups, this is not an effect of hadacidin treatment.

Table 5 compares twitch and tetanus contraction characteristics in control vs. hadacidin-treated muscles before and after the stimulation/recovery protocol. There were no significant differences in twitch or tetanus characteristics between groups either before or after the protocol. There was a trend toward decreased peak twitch force (18%) in both groups after the protocol. There were also trends toward decreased peak force and prolonged rise time of 500 ms duration test tetani in the hadacidin-treated group after the protocol, although these trends were not significant.

Response to Submaximal Twitch Stimulation.

Figure 12 shows typical spectra acquired during and after 8 minutes of stimulation at 0.75 Hz, a rate known to be within the steady-state aerobic capacity of normal rat hindlimb muscle (68). ATP depletion had no significant effect on peak twitch force during the stimulation (Figure 13A). However, the initial rate of net PCr hydrolysis was slower, and the steady-state PCr level attained during the last minute of stimulation was higher, in ATP-depleted compared to control muscles (Figure 13B). The time constant for PCr changes (τ), the final steady-state PCr level during stimulation (PCr_{SS}), and the initial rates of PCr hydrolysis ($dPCr/dt|_{t=0}=(PCr_{t=0}-PCr_{SS})/\tau$) were estimated from monoexponential fits to the PCr data from individual muscles (Table 6). These fits confirmed that PCr_{SS} was slightly higher in ATP-depleted compared to control muscles, despite the fact that the time constants for PCr changes were significantly longer in the ATP-depleted compared

TABLE 5. Contraction characteristics of test twitches and 500 ms tetani before and after tetanic stimulation-recovery protocol.

	Pre-treatment		Post-treatment	
	Control	ATP-depleted	Control	ATP-depleted
Twitch:				
Peak force (g/g muscle)	1.63±0.11	1.85±0.32	1.32±0.21	1.52±0.29
Time to peak tension (ms)	26±2	26±4	21±1	25±3
Half relaxation time (ms)	22±3	22±5	24±4	33±5
Tetanus:				
Peak force (g/g muscle)	7.38±0.30	7.17±0.18	7.44±0.26	6.60±0.53
Rise time (ms)	79±6	79±4	86±9	116±18
Relaxation time (ms)	55±3	62±6	77±3	78±8

All values are mean ± SE, n=4. Tetanus rise and relaxation times are times from 10% to 90% and from 90% to 10% of peak force, respectively. "Pre-treatment" is after hadacidin or saline injection but before the initial tetanic stimulation. "Post-treatment" is 75 minutes after the second tetanic bout.

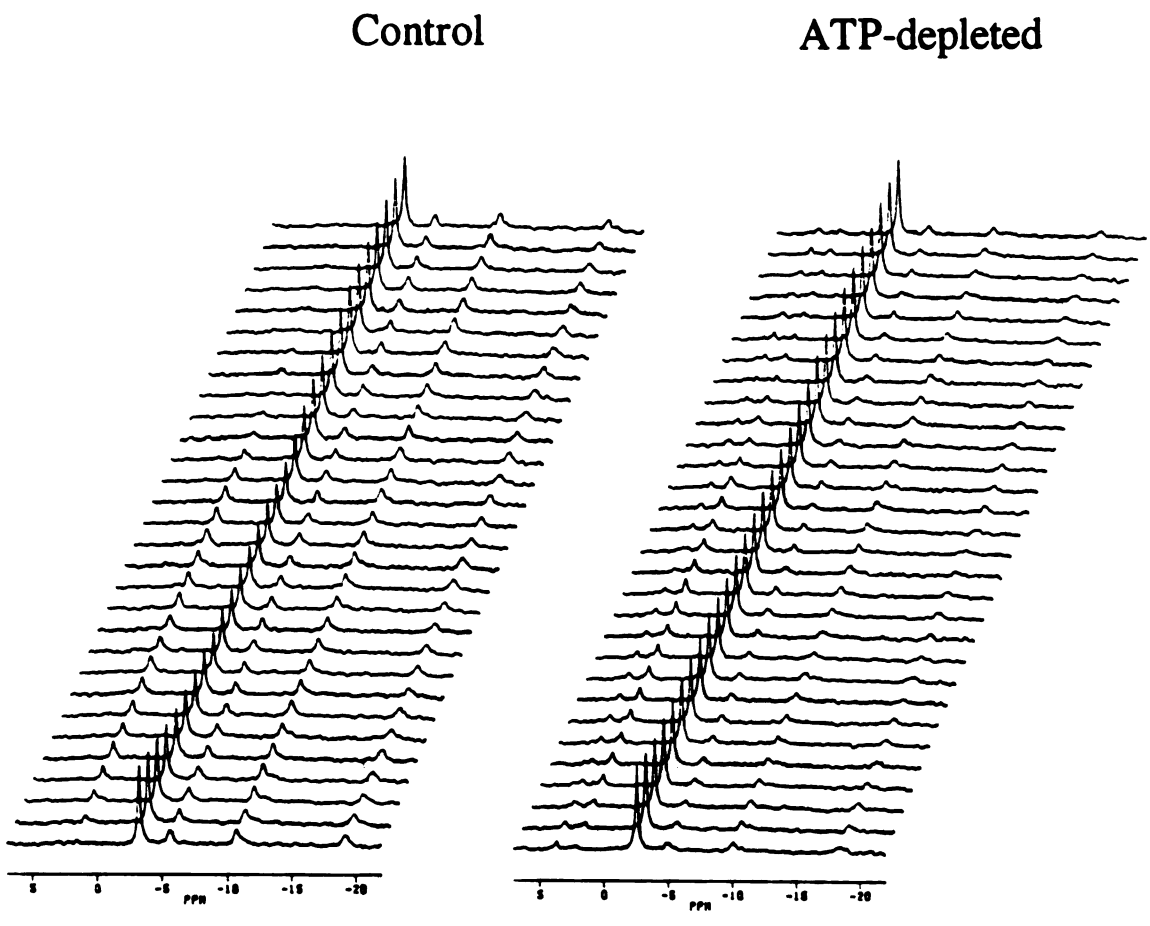

FIGURE 12. ^{31}P-NMR spectra from control and ATP-depleted rat gastrocnemius muscles during isometric twitch stimulation.

Spectra acquired before (bottom spectrum), during (next 16 spectra) and after 0.75 Hz stimulation of control (left) and ATP-depleted (right) rat gastrocnemius muscles. Each spectrum is average over 30 s (16 scans plus one dummy scan, 1.7 s interval, 7 KHz sweep width, 2K data). Peak identification is as defined in Figure 10.

to control muscles. In control muscles, τ during both stimulation and recovery averaged around 1.4 minutes, in agreement with previous studies of rat gastrocnemius muscle (104, 101). In contrast, τ in ATP-depleted muscles was over 2 minutes. Thus, the calculated initial rate of PCr hydrolysis at the onset of stimulation was 2-fold higher in control compared to ATP-depleted muscles.

Intracellular pH was initially slightly more acidic in the ATP-depleted muscles compared to controls (Table 3). Following a transient alkalinization of about 0.1 pH unit, which can be attributed to proton consumption by net PCr hydrolysis (102), intracellular pH gradually declined in both groups during twitch stimulation (Figure 13C). However, although pH remained consistently higher in the control group throughout the 8 minute stimulation period, there was a significant net acidification by the end of stimulation in the control group, but not in the ATP-depleted group. At the beginning of the recovery period, pH decreased further in both groups. This acidification was faster and more extensive in the control group, as expected from the faster and more extensive PCr change in the control muscles (Table 6). Beyond 2 minutes of recovery, the P_i peak in control muscle spectra became too broad to be consistently resolved, rendering estimation of pH impossible beyond this point. The continued presence of a resolvable P_i peak in ATP-depleted muscle is expected due to the higher P_i content of these muscles after the depletion protocol (Table 3).

The calculated free ADP content (Figure 13D) increased in both groups during twitch stimulation. However, calculated ADP was always much lower in the ATP-depleted muscles, reaching a peak level only slightly greater than the ADP level in control muscles at rest. In

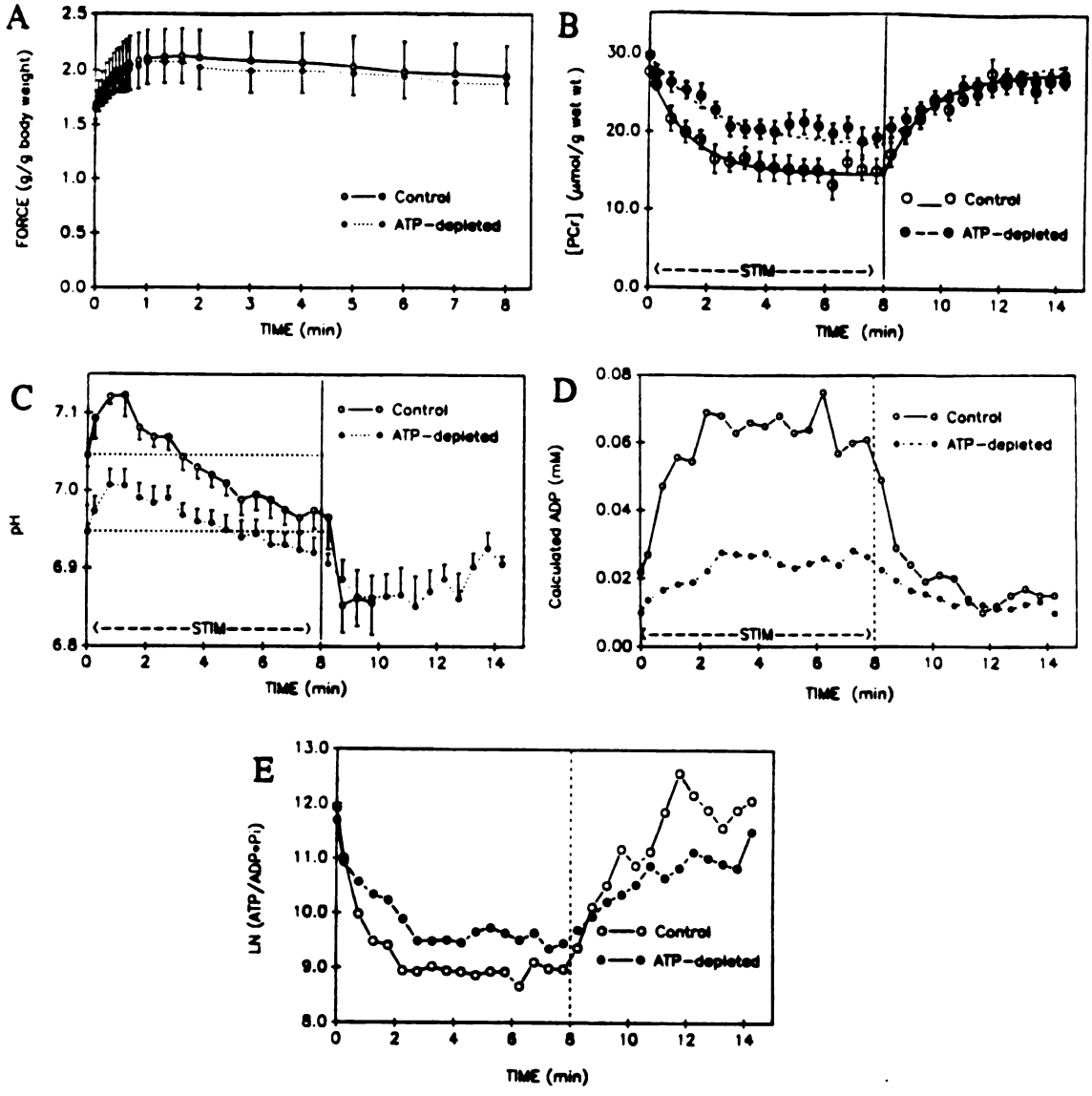

FIGURE 13. Peak isometric twitch force, phosphocreatine, pH, ADP, and phosphorylation potential in control and ATP-depleted muscles during and after final twitch stimulation.

A: Peak isometric twitch force.
B: Phosphocreatine. (PCr levels are calculated from NMR spectra as described in text. Lines in PCr plot are result of monoexponential fits to PCr changes in individual muscles (see Table 6)).
C: Intracellular pH. (Calculated from chemical shift of P_i (120)).
D: ADP. (Calculated from Equation A as described in text.)
E: Phosphorylation potential. (Calculated using [ADP] from Graph *D*.)
Muscles were stimulated for 8 minutes at 0.75 Hz as described in text. Points are means ± SE, n = 8 muscles per group.

TABLE 6. Characteristics of PCr changes during and after twitch stimulation as calculated from monoexponential fits.

	Control	ATP-depleted
Steady-state PCr during stimulation (PCr_{SS}, μmol/g)	13.8±1.4	18.3±2.4
Stimulation time constant (τ, min)	1.34±0.30	2.11±0.25 *
Recovery time constant (τ, min)	1.42±0.33	2.68±0.60 *
Initial rate of PCr hydrolysis ($dPCr/dt|_{t=0}$, μmol/g/min)	6.95±0.83	3.19±0.53 *

Values are mean ± SE, n=7 and 6 for control and ATP-depleted, respectively. Initial rate of PCr hydrolysis calculated from fits to individual muscle data, assuming monoexponential changes:

$$dPCr/dt = PCr_{SS} + (PCr_{t=0} - PCr_{SS})*\exp(-t/\tau),$$

or, by differentiation:

$$dPCr/dt|_{t=0} = (PCr_{t=0} - PCr_{SS})/\tau.$$

* Significantly different from control, $p<0.05$

contrast, phosphorylation potential ($\ln(ATP/ADP*P_i)$) was initially similar in both groups (Table 3), and the change in this parameter during twitch stimulation was only slightly attenuated in the ATP-depleted muscles compared to controls (Figure 13E).

Response to Tetanic Stimulation.

Figure 14 illustrates the much more dramatic metabolic changes observed in muscles subjected to a final bout of tetanic rather than twitch stimulation. It should be noted that in normal rat fast twitch muscle, the tetanus rate used (1Hz @ 100 ms) results in an ATP utilization rate (estimated from the oxygen cost of 100 ms tetani (68)) over 10-fold greater than that during 0.75 Hz twitch

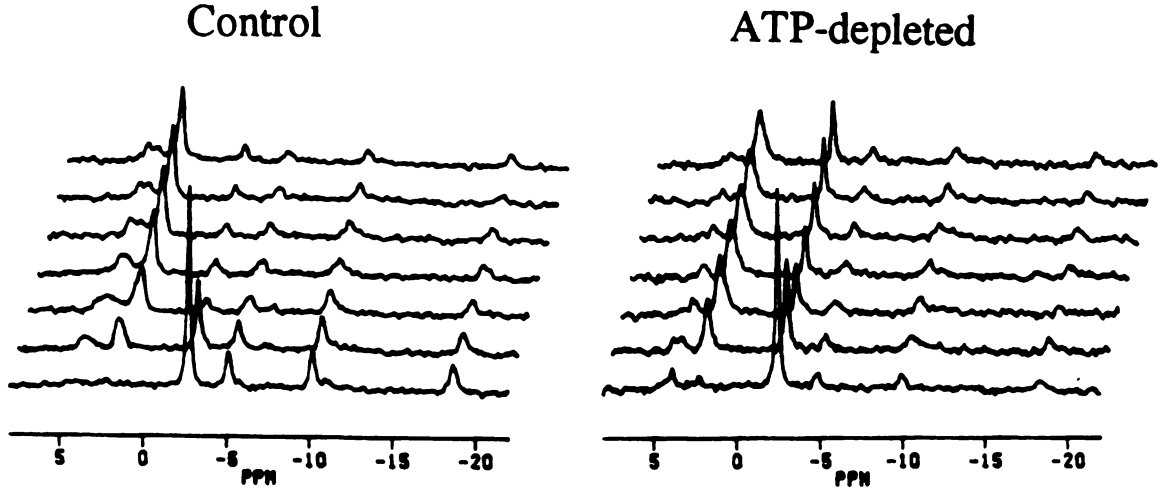

FIGURE 14. ^{31}P-NMR spectra from control and ATP-depleted rat gastrocnemius muscles during final tetanic stimulation.

***LEFT SERIES*: Control muscle.**
***RIGHT SERIES*: ATP-depleted muscle.**
Bottom spectrum is before and next 6 spectra are during 3 minutes of intermittent tetanic stimulation as described in text.
Each spectrum is average over 30 s (16 scans, 1.7 s interval, 7 KHz sweep width, 2K data).

stimulation, and therefore well beyond that which can normally be supported by aerobic metabolism.

Peak force was initially lower in the ATP-depleted muscles compared to controls, and also compared to force in the same muscles at the start of the protocol (Figure 15A). However, force in the ATP-depleted muscles increased during the first 15-20 seconds of stimulation. The apparent staircase effect in peak tetanic tension in the ATP-depleted group was similar to that observed in both groups during the second of the two initial bouts of tetani (Figure 11). The return to a monotonic fatigue profile in the control muscles, but not in the ATP-depleted muscles, after the recovery period suggests that this staircase effect is related to ATP depletion. After 20 seconds, peak force was greater in ATP-depleted compared to control muscles. Thus, total tension development during the 3 minutes stimulation period was somewhat greater in the ATP-depleted muscles.

ATP was again depleted during the final tetanic stimulation in control muscles (Figure 15B). However, there was no further change in ATP content in the ATP-depleted muscles. PCr rapidly decreased in both groups during tetanic stimulation, although the change was slightly greater in the control muscles (Figure 15C). Moreover, intracellular pH changes were remarkably attenuated in the ATP-depleted group compared to controls (Figure 15D). Over the course of the 3 minute stimulation period, a net acidification of 0.55 ± 0.08 pH units occurred in controls, compared to only 0.17 ± 0.02 in ATP-depleted muscles. Assuming a nonphosphate buffer capacity (β) of 25 μmol/g/pH unit for rat muscle (102, 4) these results can be used to estimate the intracellular acid load during stimulation, according to:

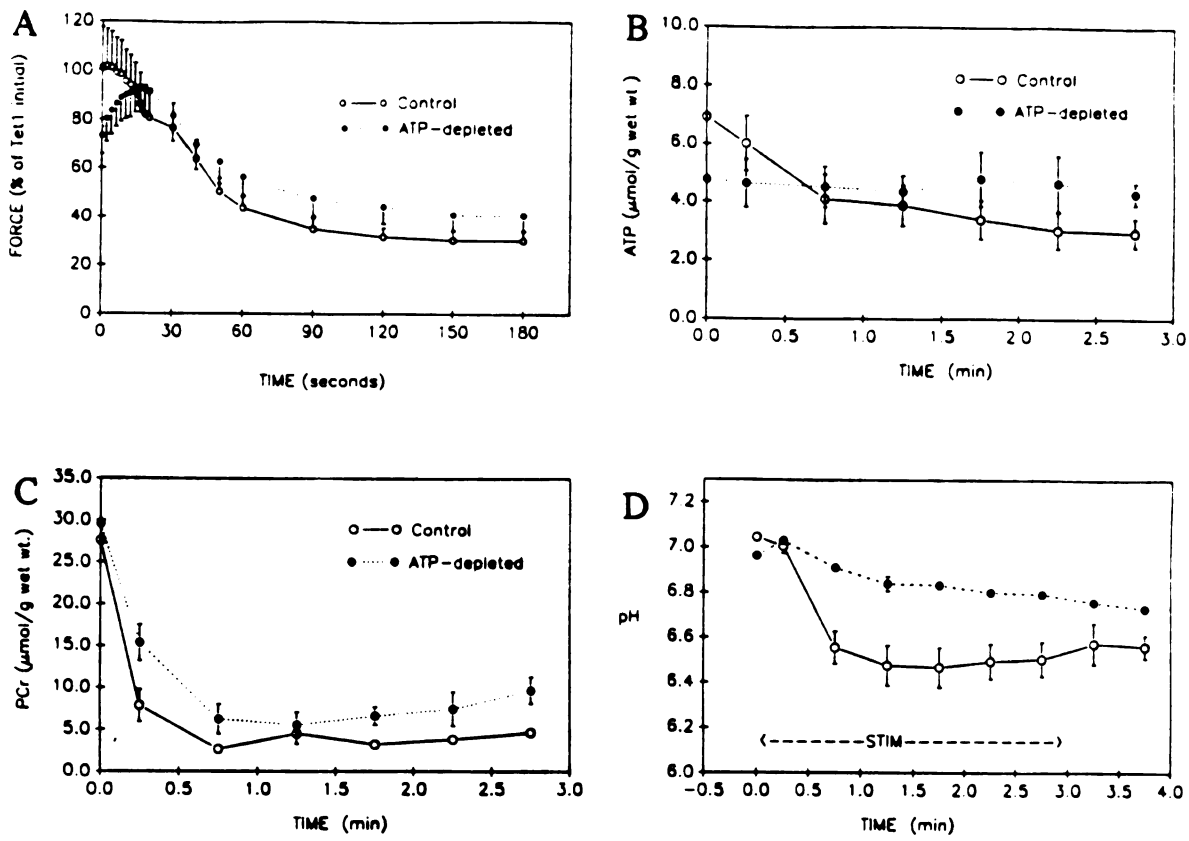

FIGURE 15. Peak isometric tetanic force, ATP, phosphocreatine, and pH in control and ATP-depleted muscles during and after final tetanic stimulation.

A: Peak isometric tetanic force. (Percent of initial force at the start of the first series of tetani applied at the beginning of the depletion protocol (see Figure 11A)).
B: ATP. (Calculated from NMR spectra as described in text.)
C: Phosphocreatine. (PCr levels are calculated from NMR spectra as described in text.)
D: Intracellular pH. (Calculated from chemical shift of P_i (120)).
Points are means ± SE, n = 5 muscles per group.

$$(C) \quad \text{acid load } (\mu mol/g) = pH * \beta + [PCr] * \alpha \quad (102)$$

The coefficient α represents the molar proportion of hydrogen ions consumed during net PCr hydrolysis and is calculated from the pK_a's of P_i and PCr (6.8 and 4.6, respectively) to be 0.69 at pH 6.50 (final pH of stimulated control muscles) and 0.54 at pH 6.79 (ATP-depleted muscles). The result of this calculation suggests that the control muscles produced more than twice as much acid (presumably lactic acid) as the ATP-depleted muscles (32 vs. 15 μmol/g) during tetanic stimulation. Applying the same formula to data from the twitch experiments produced a similar relative difference, although the acid load during this moderate stimulation was much lower (2.3 μmol/g in controls, 1.2 in ATP-depleted muscles) compared to during tetanic stimulation.

DISCUSSION

Control of Respiration.

The specific hypothesis which inspired this study was that, if muscle respiration is feedback-regulated primarily by cytoplasmic ADP, then acute depletion of ATP and total adenine nucleotide ought to result in decreased PCr at any stimulation rate. This prediction followed directly from the assumed equilibrium of the creatine kinase reaction (equation A), which is commonly used to calculate free ADP in muscle (103, 146). This specific hypothesis was apparently negated, even considering only the results of muscles at rest, before the final

stimulations (Table 3). PCr was not lower in the ATP-depleted muscles; in fact it was slightly higher than in controls. Likewise, pH in the resting, recovered muscles did not increase, but rather showed a slight decrease. Thus, the calculated ADP content in ATP-depleted muscles at rest was less than half that in the control muscles. Although we have not measured oxygen consumption in this study, it does not seem plausible that the resting oxygen consumption of the ATP-depleted muscle could be less than half that in the controls, considering that PCr levels were stable in both groups, and that intracellular pH was only slightly decreased by ATP depletion. On the other hand, the calculated phosphorylation potential was essentially identical in the two groups. Thus, the straightforward interpretation of the results in resting muscles is that phosphorylation potential rather than ADP per se is the regulated metabolic variable (104, 112, 80).

The results during twitch stimulation also clearly favor phosphorylation potential rather than ADP as the parameter controlling respiration. The twitch rate used was previously shown to be predominantly supported by aerobic metabolism in normal rat fast twitch muscle (68), a conclusion confirmed by the low acid accumulation observed in both groups in this study. During this stimulation, in which both groups developed the same force, calculated ADP in the ATP-depleted muscles barely exceeded ADP in the control muscles at rest. In contrast, changes in phosphorylation potential were similar, although not identical, in the two groups. Considering this result, together with other recent observations (100) and model calculations (125), the hypothesis that skeletal muscle respiration is controlled in a simple Michaelis-Menten fashion by cytoplasmic ADP seems clearly untenable.

However, there is an unanticipated and remarkable feature of our results which complicates their application to the simple hypothesis discussed above. The results strongly suggest that the energy cost of isometric force development was decreased by the ATP-depletion protocol.

Energy Cost.

During the twitch experiment, the estimated initial rate of PCr hydrolysis in the ATP-depleted muscles was roughly half that in the controls, despite the fact that twitch force was identical in the two groups. It is unlikely that anaerobic glycolysis made a significant contribution to ATP supply in either group, considering that pH became initially alkaline, and that even after 8 minutes only a small acid load had accumulated. Therefore, the initial rate of PCr hydrolysis provides a good estimate of the ATPase rate associated with the twitch contractions. In the control muscles, the initial rate was 6.95 μmol/g/min, which (for 0.75 Hz or 45 twitches/min) corresponds to 0.15 μmol PCr hydrolyzed/g/twitch. This compares reasonably well with the ATP cost per twitch estimated from steady-state oxygen consumption measurements in rat hindlimb muscle by Hood, et al ((68), 0.22 μmol/g/twitch, assuming P:O ratio = 3). In contrast, the initial rate of PCr hydrolysis in the ATP-depleted muscles was 3.19 μmol/g/min, corresponding to an ATP cost of only 0.07 μmol/g/twitch.

This remarkable result is confirmed by the dramatic difference in acid accumulation during tetanic stimulation. Although final force development in the ATP-depleted muscles was slightly higher, these muscles accumulated less than half as much acid as controls during this supramaximal stimulation. Furthermore, the initial rate of PCr

hydrolysis was less, and there was no further loss of ATP, in the ATP-depleted muscles. The most straightforward interpretation is that the energy cost of tetanic contractions was substantially decreased in the ATP-depleted muscles compared to controls.

A potential problem with this interpretation is the fact that the NMR spectra are recorded from only an ill-defined superficial portion of the muscle (83), whereas force is recorded from the entire gastrocnemius-plantaris group in our preparation. For example, it might be argued that the superficial fibers were for some reason less activated after the stimulation/recovery protocol, and that this problem is more serious in the ATP-depleted group, thereby attenuating the measured metabolic changes. However, three points argue against this possibility. First, and most obviously, forces were not significantly different in the two groups. Second, the metabolic results in control muscles during the final stimulation are very similar to many previous results obtained in the same rat preparation without any prior stimulation protocol (114, 113, 68, 103). Finally, the PCr time constant data is not consistent with this possiblity. PCr time constant measurements naturally reflect only active fibers, wherein PCr is changing. If the superficial, predominantly fast twitch glycolytic fibers were not contracting, then the observed time constants would necessarily reflect a greater contribution from the deeper, more oxidative fibers. Thus, the time constant, which is inversely related to oxidative capacity (104), should have decreased had the superficial fibers been preferentially inactivated.

It should also be emphasized that the apparent change in muscle energy cost could not be due to the administration of hadacidin per se.

Hadacidin was previously shown to have no effect on PCr hydrolysis or lactic acid accumulation during twitch or tetanic stimulation of rat muscles which were not previously stimulated to deplete ATP (113). This result was confirmed by our pilot experiments.

Depletion of the phosphocreatine content in rat muscle by chronic feeding of the creatine analog β-guanidinopropionate (βGPA) is also accompanied by decreased ATP and total adenine nucleotide contents (137, 42). The chronic ATP depletion observed during βGPA feeding is similar in extent to that induced acutely in these experiments. Surprisingly, supramaximal (5Hz) twitch stimulation of creatine-depleted muscles had effects remarkably similar to those observed during tetanic stimulation of ATP-depleted muscles in this study, i.e., higher steady-state force development, decreased net phosphagen and ATP utilization, and dramatically decreased acid accumulation, compared to control muscles (102). These changes accompanying creatine depletion have been attributed to increased mitochondrial content (53) and/or to altered myosin expression (118). Of course neither of these adaptations could have occurred during our acute ATP-depletion protocol. It is possible that the chronic ATP depletion which occurs during βGPA feeding is at least partially responsible for the observed metabolic changes.

Unfortunately, we know of no mechanism which can account for this effect of ATP depletion on ATP utilization. Lowered ATP use has been observed under other conditions, for example, during single prolonged isometric tetani in mouse muscles (19) and during contractions at low temperatures (126). In addition, ATPase activity has been correlated with the intrinsic speed of shortening in comparative studies of muscles from vertebrate and invertebrate animals (7). However, to our

knowledge, there is no evidence that variations in ATP content in the 4-8 mM range influence contraction kinetics in skinned fibers, or ATPase rates in isolated myosin. Another logical possibility is that the increased IMP in the ATP-depleted muscles somehow modulates cross-bridge activity. Finally, it is conceivable (70, 71), although unlikely (112), that the ATP remaining after the ATP-depletion protocol is not primarily free in the cytosol, and hence that ATP is kinetically limiting for myosin ATPase.

Previous studies have demonstrated a correlation between ATP content and the energy cost of maintaining isometric tension in different fiber types in the hamster (51). This suggests an energy cost reduction mechanism based on an increased efficiency (energy cost for maintenance of tension) rather than altered economy (energy cost for development of tension) as the terms are commonly defined (81, 67, 21). In any case, whatever the mechanism, our results suggest that high nucleotide content is an important factor supporting the high ATPase rate of fast twitch muscle. A prediction emerges from this conclusion and remains to be examined: the maximum velocity of shortening ought to be reduced in ATP-depleted muscles.

Other Effects of ATP Depletion.

ATP depletion was associated with some other interesting and unanticipated effects. First, the time constants for PCr changes during and after twitch stimulation were about 50% longer than in the control muscles. According to an electrical analog model of muscle respiration (104), PCr time constants during submaximal stimulation are inversely dependent on muscle aerobic capacity. Thus, our results suggest that aerobic capacity was significantly decreased by ATP depletion. This

could be viewed as a result of inhibition of IMP reamination, and hence of fumarate production (95), rather than of ATP depletion per se. However, this inferred decrease in aerobic capacity was not associated with greater fatigue during stimulation in the ATP-depleted muscles, apparently because the contracting ATP-depleted muscles used less ATP than did controls contracting at the same rate and with the same peak force. In this context, the smaller change in phosphorylation potential in ATP-depleted muscles during twitch stimulation is still consistent with the proposal that phosphorylation potential is the key regulator of respiration. On balance, less respiratory drive was required to maintain the lower ATP turnover in ATP-depleted muscles.

Another surprising effect of the ATP-depletion protocol was the staircase effect observed during repetitive tetanic stimulation. This effect occurred in both groups during the second of the two initial tetanic stimulation bouts, but only in the ATP-depleted group after the intervening recovery period. Therefore, the staircase cannot be a direct effect of hadacidin treatment per se. A staircase of peak force is commonly associated with repetitive low frequency twitch stimulation in fast twitch muscles, as illustrated in Figure 13A of the present chapter and in Figure 2 of chapter three. However, this pattern of potentiated force has to our knowledge never been observed to occur in a series of tetanic trains as reported here. The mechanism for this unique observation is obscure, although recent studies of twitch potentiation in mouse skeletal muscle have uncovered a possible link between this staircase effect and the level of phosphorylation of myosin light chains (124). Along this line, it is interesting to note that in rat gastrocnemius muscles chronically depleted of PCr, this twitch

potentiation disappeared (102). Inasmuch as this treatment also produced an ATP depletion similar in magnitude to that produced acutely in the present experiments, it would be of interest to determine whether this chronic ATP depletion is associated with the tetanic potentiation phenomenon reported in our acute experiments. If so, the mechanisms of the two potentiation phenomena would likely be of different origin.

Finally, there was a slight but significant decrease in intracellular pH associated with ATP depletion. This could arise by many mechanisms. One possibility is that the high IMP content in ATP-depleted muscles results in higher basal rates of glycogenolysis and lactic acid production by activating glycogen phosphorylase (18). On the other hand, it is apparent that IMP is not the sole, or even a major determinant of lactic acid production during tetanic stimulation, inasmuch as high IMP was already present at the onset of stimulation in the ATP-depleted muscles, and yet these accumulated much less acid than control muscles.

In summary, acute depletion of ATP from rat fast twitch muscle results in metabolic changes consistent with decreased ATP turnover during isometric contractions. Despite this surprising complication, the results are consistent with the proposal that phosphorylation potential, rather than ADP alone, is the cytoplasmic factor which regulates respiration in muscle.

V. SUMMARY

"Research is not a systematic occupation but an intuitive artistic vocation."

A. Szent-Györgyi, 1963 (142)

The first portion of this research addressed the problem of conflicting claims regarding the importance of the purine nucleotide cycle in aerobic energy metabolism in skeletal muscle. The experiments reported in chapter three using the drug AICAr resolved this question satisfactorily. The aerobic performance decrement attributed to PNC inhibition in previous studies (43) was demonstrated to be more likely caused by systemic effects of the particular drug AICAr being used as a PNC inhibitor in those experiments.

The significance of this finding is twofold. First, future investigations of PNC function in vivo should not rely on the drug AICAr as a PNC blocker because of these confounding effects. Secondly, the use of verifiably selective PNC inhibitors to study muscle energy metabolism now cannot be questioned on the grounds of interference with aerobic pathways on the basis of the previous AICAr report (43). This conclusion directly impacted the design of the principal research plan for this thesis, relying as it did on the use of the selective PNC inhibitor hadacidin to produce an in vivo model of ATP depletion in muscle.

The primary question being addressed in this dissertation was: Is kinetic control (by amount of the phosphate acceptor ADP) of the rate of oxidative phosphorylation sufficient to explain the regulation of mitochondrial respiratory rate in contracting skeletal muscle?

The results reported in the previous chapter clearly indicate that control by ADP alone is unlikely, and that the theory of thermodynamic control via the regulatory parameter [ATP]/([ADP]*[P_i]) is more consistent with these observations.

In addition to this main outcome, some other rather surprising and unexpected observations arose from these studies. First, depletion of ATP was associated with an apparent reduction in energy cost of muscular contractions. If the association is confirmed as a true cause-effect relationship, this result may be of value in interpreting the findings of previous studies in which ATP depletion was but one of several variables associated with an experimental manipulation (e.g. 102, 53, 118). Testing of the prediction of reduced maximum shortening velocity in ATP-depleted muscles should help resolve this question. Further studies directly measuring oxygen consumption in ATP-depleted muscles are also suggested by these results.

The apparent reduction in oxidative capacity, as inferred (104) from the lengthened time constants of PCr changes in ATP-depleted muscle, is another interesting but as yet unexplained finding. The reduction in acid accumulation in ATP-depleted muscle, suggesting reduced glycolytic/glycogenolytic activity, is also consistent with reduced energy cost of contraction. Both of these issues should be examined to determine the mechanism by which ATP depletion produces these results. For example, examination of mitochondria from acutely ATP-depleted muscle may reveal if oxidative capacity is in fact reduced, thereby providing another test of the predictive capacity of the circuit model (104). Studies of glycogen content in ATP-depleted muscle would help determine if the decreased glycolytic flux is due to substrate

limitation or is more likely caused by enzyme inhibition.

Finally, the staircase or potentiation of tetanic force observed in these studies is a phenomenon that has not been previously reported in the muscle literature. The discovery of the potentiation phenomenon in the second of two tetanic trains separated by only a short rest interval was a serendipitous result of trying a varierty of methods to achieve the goal of maximum ATP depletion. In the very first steady state studies of in situ gastrocnemius muscle over 50 years ago, Sacks, Sacks and Shaw happened upon an interesting result in a similar fashion (131), commenting that: "The two rates were selected arbitrarily, but the results obtained proved the choice to be a happy one." The tetanic potentiation observed in the present study was clearly not a result of hadacidin administration, but rather a more generalized effect apparently associated with ATP depletion.

In summary, the depletion of ATP in skeletal muscle produces changes in phosphorus metabolites that are not consistent with acceptor control of oxidative phosphorylation. These results can, however, be explained satisfactorily by the mechanism of control by the phosphorylation potential of cytoplasmic ATP.

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