FROM CROSSBRIDGES TO METABOLISM: SYSTEM BIOLOGY FOR ENERGETICS

Martin J. Kushmerick*

INTRODUCTION

AV Hill's studies of muscle mechanics and heat production defined muscle energetics. Myothermal analysis relied on the fact that the total energy change between two states of a muscle is equal to the sum of the heat output and the work done. To interpret this application of the first law of thermodynamics as chemical changes in muscle one must identify the metabolic reactions, know their molar enthalpies, quantify their extents of reaction, and of course measure the heat produced and the work done (32). ATP splitting was suspected as the chemical driving force for these interactions that produced mechanical and thermal outputs, but changes in ATP content could not be measured directly in living muscle. Earlier Lundsgaard showed decreases in phosphorylcreatine (PCr) did occur in muscle but that PCr was not a substrate for myosin. Biochemical and physiological information appeared to be in conflict. By inhibiting creatine kinase, Davies's lab did show directly a splitting of ATP into ADP and inorganic phosphate in a single contraction of frog muscle (3). Understanding of muscle rapidly evolved with analyses of heat, work, chemical changes, and economy and efficiency brought to fruition by a number of laboratories within the context originally defined by Hill (reviewed in (33)).

The first goal of this paper is to define a set of biochemical rules that are equivalent to those of thermodynamics in the sense of accounting for muscle energetics. These rules are not a priori predictable from first principles of thermodynamics because the mechanisms involved follow chemical principles; the pathways and reactions used were selected by evolution. Our analysis reveals biochemical-based rules and integrates them quantitatively into a comprehensive model useful for basic and translational research. This work forms an outline for a "system biology for energetics" meaning that a synthesis of mechanisms from the molecular to tissue and organ is feasible. This synthesis is quantitatively given by a set of rules and equations. The burden of this

* Martin J. Kushmerick, University of Washington, Seattle, Washington, 98195.

approach is to quantify the rate and extent of the critical reactions, measure their stoichiometry, quantify their fluxes and describe the mechanisms quantitatively by kinetic equations. We will see that the central reaction of the crossbridge, chemomechanical splitting of ATP, is necessarily and mechanistically connected to the regulation of metabolic pathways generating ATP; this regulation in turn influences crossbridge function through the operation of metabolic pathways, which alter the concentrations of ATP, ADP, Pi and pH and thereby influence crossbridge force and kinetics. These interacting mechanisms are central features of the system biology approach outlined here.

The evolution of these ideas began with the work of Lipmann (21). He used the phrase "high energy phosphate bond" to point out that, with all the intricacy of metabolic pathways, there are very few molecules involved in energy transducing mechanisms, the most significant being ATP. From our vantage point of energy balance, metabolic pathways are biochemical structures whose regulation controls the ATP synthesis. Energy transductions in muscle are carried out by molecular electro-chemical and chemo-osmotic machines and chemo-mechanical motors schematically shown as modules connected through a common intermediate ATP (Fig. 1). These modules synthesize or dissipate ATP, the cell's source of chemical potential energy, in a way that couples chemical energy to the metabolic, electrical, osmotic and mechanical work. Although a small amount of ATP is stored in the cell (on the order of 6-8 mM), ATP must be produced by metabolism in a steady state of work performance. The reason is that typical rates of ATP utilization in active muscle (on the order of 0.5 mM per sec) would rapidly exhaust the muscle's source of chemical potential energy in ~ 10 sec. We know that steady states of metabolic activity are possible during exercise over many minutes to hours. The question is what are the rules and mechanisms that match supply to demand?

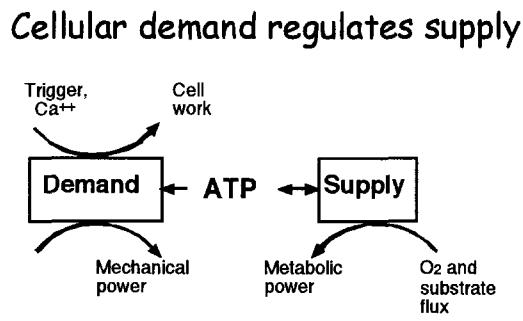

Figure 1: Diagram of the two major components in energy balance in muscle: demand of ATP utilization and supply by its synthesis. Both modules are coupled by functions of ATP.

My second goal here is to demonstrate methods we developed to reveal the mutual interconnections between energy metabolism and crossbridge function. Instead of studying the details of myothermal balances (which remain a valuable experimental approach with high time resolution and accuracy), we bring analyses of chemical fluxes coupled to mechanical output and the regulation of metabolic ATP synthesis to the forefront. I envision an integration of mechanisms from the kinetics of ATP hydrolysis in a cycle of attachment and detachment of crossbridges to the regulation of mitochondrial oxidative phosphorylation and cytoplasmic glycogenolysis in full biochemical detail.

To build this integrated view we need to begin with the essential reactions that are involved and focus on their stoichoimetric relations. Understanding of the implications of these reactions lead us to novel methods for measuring critical chemical

fluxes. These reactions produce or take up Pi, ADP and H^+ by stoichiometric rules of chemistry. It is important to recognize that these molecules serve to modulate crossbridge activity and kinetics as well as control metabolic fluxes. Pi affects kinetics of strong attachment transitions. H^+ effects are distributed, and may be modeled best as influencing the strength of myosin to actin interaction because the major part of the binding site energy is ionic. Hence, in the muscle, cell crossbridge function cannot be divorced from intermediary metabolic pathways involved in the regeneration of ATP as depicted in Figure 1.

CONTRIBUTIONS TO ENERGETICS BY 31P NMR SPECTROSCOPY

Much progress toward the goal of a non-invasive measure of muscle cell energetics has been achieved by applications of ^{31}P magnetic resonance spectroscopy (for reviews see (23, 28)). Typical results are displayed in Fig 2 which are taken from our work on human forearm muscle (1). With the onset of mechanical activity in human and other animal muscles, PCr content decreases (down arrow on PCr peaks), and the content of inorganic phosphate (Pi) increases (up arrow on Pi peaks). During aerobic recovery from moderate activity, there can be an apparently simple exponential time course of re-synthesis of PCr and decrease in Pi with a return of normal intracellular pH (25). These observations are consistent with feedback control of cellular respiration by ADP concentration. Thus measurements of PCr decline and re-synthesis can provide a means for characterizing the components of the muscle energy balance to define directly the functional state of the muscle. These studies forced a revision of metabolic control by these metabolites because:

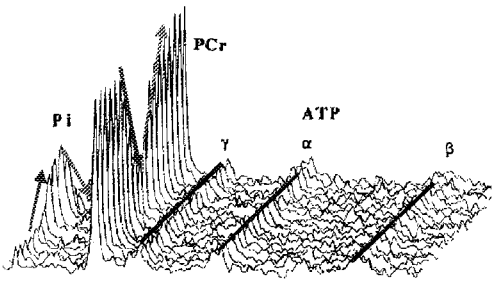

Figure 2: Plot of every 10th spectrum obtained serially during a ^{31}P NMR experiment. The peak identities from the left are: Pi, PCr, and the three peaks of ATP. Down and up arrows indicate changes measured during muscle activity.

- the concentration of Pi is on the order of 1-3 mM, not 10 mM or higher, as defined by prior analyses of muscle extracts,
- the concentration ADP is on the order of 0.02 mM, not the 0.4 to 0.6 mM as measured in muscle extracts that include a large amount of ADP bound to actin and other proteins, and
- the range of pH can be as large as a full pH unit in the acid direction from a normal pH of ~ 7.

We defined an experimental approach for separating ATP-utilization from ATP-synthesis by a quantitative metabolic stress test in normal volunteers (1, 2). This test used a comparison between normal and ischemic contraction and common aerobic recovery protocol. This approach allowed separation and quantification of the ATP-utilization phase from ATP re-synthesis in the human muscles. In the ischemic protocol the initial rate of PCr decline equals the ATPase flux for that experiment. Release of the pressure cuff allowed aerobic recovery by restoring blood flow and oxygen delivery

with initiation of mitochondrial oxidative phosphorylation. The time course of PCr resynthesis is accurately fitted by a monoexponential curve which describes the capacity for mitochondrial ATP synthesis by oxidative phosphorylation (24). PCr content returns to the resting, pre-stimulation level. Recovery kinetics of PCr following exercise are in accord with a simple model of respiratory control (4).

RULES FOR STOICHIOMETRY OF METABOLIC REACTIONS

Here we consider some details of the chemistry of relevant reactions in energetics; full details are available (17). The first kind of stoichiometric relationships are those defined by the laws of chemical combination and mass conservation. The fact that they are found in muscle is the product of evolution rather than deductive reasoning from first principles. ATP splitting is tightly coupled by crossbridge motor to perform work; other ATPases are similarly coupled to electrical and osmotic work of ion transport:

ATP hydrolysis: $ATP = ADP + Pi + \alpha H^+ + work$ Eqn 1

The large Gibbs energy for this reaction is due largely to proton binding under intracellular physiological conditions (10). The value of the stoichiometric coefficient α is 0.74 at pH 7.0.

The second reaction of importance is that catalyzed by creatine kinase:

$PCr + ADP + \beta H^+ = ATP + Cr$ Eqn 2

Note the term βH^+ is on the left which means that protons are taken up as the reaction advances in the direction written. The value of β at pH 7.0 is 0.93 in the cytoplasm. The absolute magnitude of β decreases significantly at lower pH values such as occurs in heavy muscular exercise or tissue hypoxia. Of course both reactions occur in the cell, so that their combined action is given by their summation, which is known as the Lohmann reaction:

$PCr = Cr + Pi + \gamma H^+$ Eqn 3

The algebraic sum $\alpha + \beta$ defines the proton stoichiometric coefficient γ. The value of γ at pH 7.0 is -0.19; this means that net PCr breakdown throughout the course of contraction causes the cytoplasm to become alkaline. The reverse occurs in ATP resynthesis. Notice the stoichiometric coefficients (Greek symbols) for H^+ are not unity nor are they constant; they depend on the concentration of cations and pH of the cytoplasm (17).

APPLICATIONS OF STOICHIOMETRY FOR MEASUREMENT OF PATHWAY FLUX

These stoichiometric rules allow the time course of chemical changes to be made in terms of specific components of energy balance and can extend the interpretation. The first example is the creatine kinase reaction. Because it is coupling to ATPase by the Lohmann reaction, these stoichiometric relationships establish the validity of the interpretation of PCr changes as ATPase and ATP synthesis fluxes. A second example is the ability to measure the intracellular buffer capacity (5, 6). During the first several seconds of the abrupt onset of exercise the intracellar pH rises from 7.0 to ~ 7.1. During the initial ~ 20 sec at the onset of contractile activity, these stoichiometric relationships predict an alkalinization, provided that the only reactions occurring are those given in Eqn 1 – 3 as depicted schematically in Fig. 3. The upper curve of the left panel shows

the predictions for the Lohmann reaction with alkalinization occurring until the onset of recovery. The lower curve of the left panel depicts the observed pH change. The difference indicated by the vertical lines represents the contribution to glycolysis. We can use these stoichiometric relationships to assess pH buffer capacity by the measurements depicted in the right panel. The decrease in PCr while pH goes alkaline are defined by the dashed lines in the figure. Because the only net reaction occurring at this time is PCr breakdown to Pi and Cr at a significantly higher rate than at rest, the stoichiometry of this reaction results in uptake of H^+. The buffer capacity, defined as the moles of H^+ produced per unit pH change, is measured from these relationships; standard units are

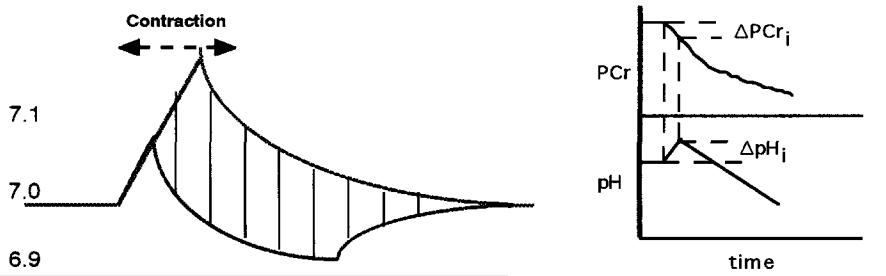

Figure 3: Diagram for method of measuring intracellular buffer capacity. The left panel shows simulations of expected pH calculated from the stoichiometric relationships in text; in the lower curve the measured pH is depicted and the vertical bars represent the difference. The right panel depicts the measurement of initial change in PCr and pH used to define intracellular buffer capacity; see text.

Slykes, mM H+ added per unit pH change; it is measured by the following equation:

Non-Pi buffer capacity = $\gamma \bullet \Delta PCr_{initial}/\Delta pH_{initial}$ Eqn 4

The total buffer capacity is the sum of the non-Pi buffer capacity and that due to the changing Pi concentration. This strategy was used to measure glycogenolytic flux and to analyze the turning on and off of glycolysis and glycogenolysis (7, 8). This method uses the stoichiometric coefficients as described above and full details are published (5, 6). Validation of this method was shown by direct measurements of lactate produced in muscle (14).

RULES FROM MASS AND ENERGY BALANCE

Energy balance can be described by a few simple rules on conservation of energy expressed in terms of biochemical changes observed by ^{31}P NMR spectroscopy and application of these stoichiometric rules:

1. ATP provides the energy for all forms of muscle work.

This rule is evident from the prior discussion and Lipmann's insight into the central role of ATP.

2. Creatine kinase functions as a biochemical capacitor.

A biochemical capacitor is a generalization of a buffer reaction analogous to electronic circuits where capacitors store energy. The creatine kinase reaction is a biochemical capacitor for ATP and ADP and, as such, this reaction is centrally important for muscle energetics as already described (19, 26, 31, 34). The evidence shows the CK reaction operates near equilibrium in muscle cells (15, 22, 29). The function of a biochemical capacitor is to buffer ATP and ADP concentrations against larger changes in

concentration that would otherwise occur due to bursts of ATPase or ATP synthesis fluxes. In so far as ADP is a metabolic control signal, buffering also attenuates the magnitude of the signal and slows the response of the system. Creatine kinase does depart transiently from equilibrium at the onset of contraction but not more than ~ 25% from its equilibrium value; the free energy cost of this is small, ~RTln(1.25).

3. The sum of the coupled ATPases sets the demand side of the balance and defines energetic states.

The major extent of cellular ATPases occur during the mechanical output. The sum of all of these defines the energy demand side of energy balance.

4. The products of ATPases provide control signals for energy balance.

ADP and Pi are products of ATPases with ADP being strongly buffered as described above. Both molecules are substrates for mitochondrial oxidative phosphorylation with ADP thought to be the primary controller as discussed above. The results obtained from animal and human skeletal muscle are quantitatively consistent with an ADP feedback model. Thus ATPase signals its own resynthesis and this regulation is central to the integral coupling of crossbridge function to metabolic generation of ATP.

5. The products of ATPases and ATP synthesis modulate crossbridge function.

Concentrations of ATP, Pi, ADP and pH all influence crossbridge function. As indicated above major changes in concentration occur in Pi, typically from ~ 2 to 25 mM in strenuous activity. pH changes of 0.5 unit or more during exercise (3-fold in concentration of H+) are common. Changes in ADP occur from ~ 20 to 200 μM. These variations in metabolite concentrations are sufficient to decrease force by altering the population of the AMADPPi and AMADP states of the crossbridge (11, 12, 27). Moreover differences in sensitivity of muscle phenotype to pH are known (9).

QUANTITATIVE MODEL FOR ENERGY BALANCE

It is straightforward to translate the stoichiometric principles and the rules for energy balance into a simple model of muscle bioenergetics (16, 18). Such a scheme is given in Fig 4 that gives details on the constituent mechanisms involved grouped as interacting modules; fig. 4 is a more detailed view of fig 1. The left lobe of this diagram represents the ATPase, depicting the predominant crossbridge ATPase. Activity begins by neural signals and transient Ca^{2+} increase. The right lobe represents the biochemical capacitors, vertical lobes represent the ATP synthesizing modules: oxidative phosphorylation at the top and glycolysis and glycogenolysis at the bottom. All of the molecules in the central ring are critical for these interactions as reactants, products and signals.

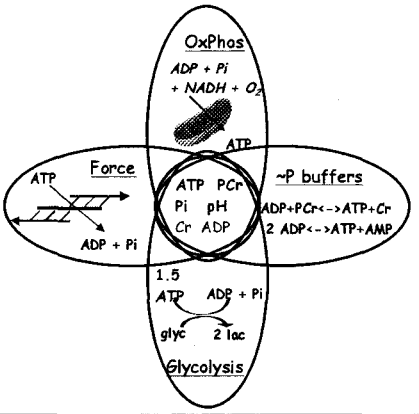

Figure 4: Diagram of intersections for components of energy balance; see text.

Energy balance can be stated mathematically by the following differential equation, which has only three terms defining the rate of change of ATP during

contractile activity: a lumped ATPase, an empirical term for control of mitochondrial oxidative phosphorylation, and a term for the creatine kinase buffer. In the discussion that follows, for simplicity we will ignore the details of glycolysis which are available (20). The kinetic function for oxidative phosphorylation is a Hill function of ADP determined empirically (13). Creatine kinase activity is determined solely by the concentration of its substrates and products; a full set of kinetic relationships, based on *in vitro* kinetic constants, were applicable to muscle (Schimerlik & Cleland, 1973) as we reported (McFarland *et al.*, 1994).

$$\frac{dATP(t)}{dt} = -kATP(t) + OxPhos(t) - \frac{dPCr(t)}{dt} \qquad \text{Eqn 5}$$

Simulations like those in Fig. 5 result by integrating Eqn 5 with parameters obtained from energy balance experiments on human limb muscle (18). The simulation in Fig 5 begins with k at a value for resting muscle. At 210 sec, the rate increases as measured for twitch stimulation and is steady until 300 sec when it returns to resting. The simulation was made by numerical approximation of these relationships: for each time step, ATPase decreased [ATP] according to the first order term, -k•ATP(t). CK flux was adjusted and new concentrations of ATP, PCr, ADP and creatine were calculated. ATP synthesis for that time step was calculated and the process was iterated for small time steps. The simulation reproduced the time courses of chemical changes observed.

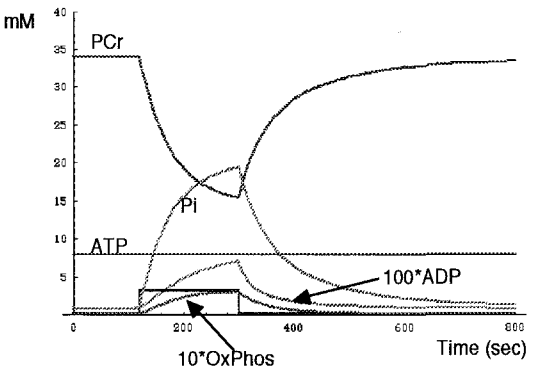

Figure 5: Simulations of chemical changes predicted by a model for energy balance. Ordinate scale is concentration of metabolites in mM or flux in mM/sec; abscissa is time in sec. 100 sec of rest is followed by 200 sec of elevated ATPase flux, then by recovery. The ATPase curve is the rectangle. The curve for ADP is multiplied by 100; the curve for oxidative phosphorylation is multiplied by 10.

FITTING EXPERIMENTAL DATA TO THE ENERGY BALANCE MODEL

This model for energy balance can be used for data analysis (30). Two further examples from consecutive measurements on one subject is displayed in Fig 6. The panel on the left represents a series of ischemic isometric twitches and the panel on the right shows the normoxic series of twitches. For each experiment, integration of spectral peaks like that shown in Fig 2 gives the data shown in the closed symbols. The lines drawn are the model fits using the equations and parameters used in the simulation in Fig 5 with the following adjustable parameters: resting and active ATPase and ADP concentration for half maximum oxidative phosphorylation. Thus model is adequate to explain the data for rest-contraction-recovery transitions in ischemic and normoxic contractions in human muscle. For both experiments the mitochondrial capacity for

oxidative phosphorylation was 0.45 mM ATP/sec, resting ATPase was 0.03 mM ATP/sec, and active ATPase was 0.41 mM ATP/sec. The apparent "Km" of ADP was 15 μM. The time course of the component metabolic fluxes is also calculable from the fitted results. The conclusion is that the

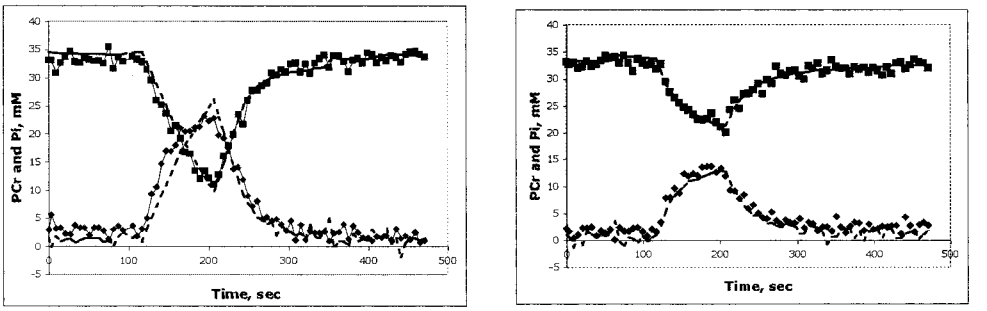

Figure 6. Examples of data and model fits using an energy balance model. Two serial experiments on one subject are displayed. The symbols are data obtained from ^{31}P spectra from the anterior compartment muscle of the human leg; PCr boxes and Pi diamonds. The protocol was 120 sec of rest; 90 sec of twitch contraction followed by recovery. The muscle was ischemic during the twitches in the left panel and normoxic in the right panel. For both, recovery occurred with normal blood flow. The lines drawn through or near the data points are the curves fitted by an energetic model (30).

model, obviously incomplete in many details including the absence of glycolytic ATP synthesis, nonetheless does provide an adequate description of the chemical events in brief bouts of exercise. These simple analyses show that this basic explanation of the reactions and components of biochemical energy balance depicted in Figs. 1 and 4 is largely correct, because the equations used, which embody current understanding of these processes, correctly account for experimental data.

CONCLUSIONS

The pathways for metabolic generation of ATP and crossbridge and other molecular machines that use ATP are necessarily connected because both sets of molecular modules share common metabolites, specifically ATP, ADP, Pi and pH. These metabolites act to modulate the flux in the mechanisms for energy demand and supply. The products of the ATPases are signals regulating the flux of ATP synthesis. The results of ATPase and ATP synthesis modulate metabolites that influence crossbridge kinetics and force generation. There are only a small number of critical chemical reactions that are necessary to advance quantitative understanding of these processes. The stoichiometric inter-relationships, defined by chemical rules, thus enforce a degree of simplification onto the system. The reason is the constraints imposed by the constancy of the internal composition, mass balances, stoichiometric rules and shared pH environment must follow ordinary conservation rules for mass and charge balances. We argue that these principles permit the obtaining of basic information concerning the organization of muscle energetics and the discerning of simple yet surprisingly powerful rules governing function. Moreover, they offer bases from which strong inferences can be made to

integrate mechanisms as diverse as molecular crossbridge motors, metabolic fluxes generating ATP, cell respiration, and muscle oxygen consumption.

Acknowledgements:
This work was supported by grants from the US National Institutes of Health (AR 41928 and AR 36281) and from the National Space Biomedical Research Institute (MA00212).

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DISCUSSION

Rall: You stated that contracting human skeletal muscle in vivo has an ATPase during contraction 15 fold higher than at rest. But in frog skeletal muscle at low temperature, the rate of energy liberation during contraction increases more than 100 fold. Why is there the difference between human and frog muscle?

Kushmerick: The range of contraction to resting flux referred to the specific examples in my presentation, where there was a 15-fold ATPase during contraction as compared to the resting state. Bear in mind that my experiments used isometric twitches at 3 per sec, not tetanic stimulation as the frog muscle to which you refer were stimulated. Twitch tension is about 10% of a maximal voluntary contraction, which is comparable to a tetanus. Moreover the initial heat plus work rate in frog muscle tetanic stimulation is higher than the steady state rate whereas my results referred to an average rate over 90 sec of the twitch stimulation. The energy fluxes are rather comparable, and I did show a table showing that my results with intact human leg muscle (crossbridge and ion pumping ATPases) were about twice those from skinned human fibers (He et al., Biophys. J. 79: 945-961, 2000; Stienen et al., Physiol. 493: 299-307, 1996) when compared on the basis of integral of tension times time per unit strain.

Gonzalez-Serratos: Have you considered in your model that a substantial amount of the ATPase activity is due to the sarcoplasmic reticulum ATPase, Na^+ / K^+ ATPase pumps etc?

Kushmerick: The model and simulations described lump all ATPases together, including the Ca^{2+} transport in SERCA. Best estimates suggest that ion transport ATP costs are on the order of 40% of the total. I consider the total ATPase occurs during the mechanical output of the twitch because that is the time the crossbridges are splitting ATP and because of the evidence from mouse muscle that cytoplasmic free Ca^{2+} is returned to baseline before the peak of twitch force (Hollingworth et al., J. Gen. Physiol. 108: 455 - 469, 1996). Precise alignment of the time courses of the separate ATPase mechanisms matters little because they occur on a sub-second time scale whereas the relevant time scale for metabolism is on the order of tens of seconds.

T. Yamada: How about pH_i changes based on the chemical shift of P_i? Under the resolution of spectra you obtained, do you see any correlation between the metabolic and mechanical changes you mentioned and the pH_i changes?

Kushmerick: Due to time limitations, I did not discuss any results measuring pH, but as described in the text these occur and have consequences. pH can be measured with a precision of better than 0.1 pH unit and sometimes with a precision of ~ 0.02 pH unit. The text of my paper discusses some of the more important metabolic consequences. The major one is acidification influences the position of the creatine kinase equilibrium, and for the same change in PCr reduces the concentration of ADP as compared to what it would have been without the pH decrease. Another consequence derives from the initial pH rise, due to net PCr splitting without other confounding reaction. This transient pH rise allows us to calculate the pH buffer capacity for each experiment, and to use that information to compute the extent of glycogenolytic and glycolytic ATP production. These and other effects are complex, and this is one of the reasons why it is necessary to make quantitative models of the processes occurring, including Pi, ADP and pH effects on crossbridges, on Ca^{2+} transport and in signaling metabolic pathways to advance our understanding of the effects of pH change.

Brenner: What about effects of compartments in cells/tissues as effects of Pi /ADP etc on crossbridges are derived?

Kushmerick: The notion of compartmentalization of low molecular weight metabolites in the cytoplasm has become popular again, but I maintain the importance of metabolite compartmentalization is over-emphasized and the effects are likely smaller than some imagine for the following reasons. This statement of course does not apply to organelles surrounded by semi-permeable membranes, the definition use for compartmentalization. Many enzymes are bound to cellular structures or are large enough to suffer restrictions to diffusion, but the small metabolites including solvent water have been found to have diffusivities on the order of one quarter to one half that of simple solutions at comparable ionic strength. We argued the case for PCr, ATP, ADP and creatine in great detail (Meyer et al., Am. J. Physiol. 246: C365-C377, 1984). Creatine kinase is definitely localized in most interesting manners (Wallimann et al., Biochem. J. 281: 21-40, 1992), but that does not mean that its reactants and products are not freely diffusible. This statement is based on NMR methods, diffusive mobility and electrophoretic mobility of

solutes injected into cells and the fact that biochemically measured enzyme activities and concentrations account for measured fluxes in cells. Most metabolites and solutes of interest have concentrations much larger than the concentration of enzyme sites to which they bind. Definitely Ca^{2+} is a major exception to this statement because of its low concentration (even at the height of its release), high concentration of binding sites and active transport into SERCA. There must be concentration gradients, for example between the myofibril and mitochondria, because there is a finite flux, on the order of 0.1 to 1 mM/sec in active muscle, but remember that the distances involved are less than a few microns. My rule of thumb for ordinary small molecular weight solutes that diffusion equilibration occurs over 1 μm in about a millisecond, and this rule suggests only a few or a few tens of μM gradients are possible, but this does not justify the notion of compartments of metabolites.