The metabolic implications of intracellular circulation

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Two views currently dominate research into cell function and regulation. Model I assumes that cell behavior is quite similar to that expected for a watery bag of enzymes and ligands. Model II assumes that three-dimensional order and structure constrain and determine metabolite behavior. A major problem in cell metabolism is determining why essentially all metabolite concentrations are remarkably stable (are homeostatic) over large changes in pathway fluxes—for convenience, this is termed the [*s*] stability paradox. For muscle cells, ATP and O_2 are the most perfectly homeostatic, even though O_2 delivery and metabolic rate correlate in a 1:1 fashion. In total, more than 60 metabolites are known to be remarkably homeostatic in differing metabolic states. Several explanations of [*s*] stability are usually given by traditional model I studies—none of which apply to all enzymes in a pathway, and all of which require diffusion as the means for changing enzyme–substrate encounter rates. In contrast, recent developments in our understanding of intracellular myosin, kinesin, and dyenin motors running on actin and tubulin tracks or cables supply a mechanistic basis for regulated intracellular circulation systems with cytoplasmic streaming rates varying over an approximately 80-fold range (from 1 to >80 $\mu m \times sec^{-1}$). These new studies raise a model II hypothesis of intracellular perfusion or convection as a primary means for bringing enzymes and substrates together under variable metabolic conditions. In this view, change in intracellular perfusion rates cause change in enzyme–substrate encounter rates and thus change in pathway fluxes with no requirement for large simultaneous changes in substrate concentrations. The ease with which this hypothesis explains the [*s*] stability paradox is one of its most compelling features.

metabolic regulation | oxygen delivery | oxygen regulation | intracellular perfusion | intracellular diffusion

Two Models and Research Approaches in Cell Metabolism and Regulation

It is a rule of thumb in biology that many physiological and molecular functions are the sum of individual processes linked in sequence; in isolation, many such individual processes have no clear functions at all. How such systems are designed and regulated have presented perplexing problems to both biochemists and physiologists. Integrated function is often evaluated by comparing changes in flux through the pathway *per se* with changes in concentrations of substrates and products of individual enzyme reactions within the pathway. Two guiding paradigms or frameworks (for convenience we will term them model I and model II) have guided these evaluations. Although rarely stated, the implicit assumptions in model I studies are that simple "solution chemistry" rules apply to the cell/tissue as a whole, that changes in rates of enzyme–substrate or protein–ligand interactions are generally diffusion dominated, and that cell behavior can thus be considered to be similar to that expected for a watery bag of organic materials. For model II studies, the starting point is structure—the microanatomy of the inside of the cell. These studies recognize that cells are filled with organelles, membranous networks, microfilaments, microtubules, channels, pumps, and motors, and that movements (not dead-still solutions as in formal model I assumptions) dominate processes inside of cells. In short, model II approaches explicitly assume that three-dimensional order and structure constrain metabolite behavior and that metabolic regulation theory has to incorporate this information to realistically describe *in vivo* processes. This polarization can be illustrated by considering a major, so far unsolved problem and paradox in the current literature; namely, that essentially all metabolite concentrations are remarkably stable (homeostatic) over large changes in pathway fluxes (1).

Phosphate Metabolite Homeostasis in Human Skeletal and Cardiac Muscles

One of our own recent noninvasive ^{31}P magnetic resonance spectroscopy (MRS) studies (2) clearly illustrates the situation. In gastrocnemius muscle, during exercise requiring up to 40-fold changes in ATP turnover rates, the concentrations of ATP are stable throughout the rest–work experimental protocols. The concentrations of phosphocreatine (PCr) and P_i change as linear functions of work, but these changes are still much smaller than the change in work (about 3-fold compared with the 40-fold increase in ATP turnover rate). Interrogated simultaneously in soleus muscle, these changes in [PCr] and [P_i] are found to be less than in gastrocnemius, whereas [ATP] and [ADP] are again stable in all states examined (2). Similar MRS studies of human (3) and dog (3) heart indicate a metabolism so well regulated that change in cardiac work is achieved with even less perturbation of MRS "visible" phosphate metabolites than in skeletal muscles.

Metabolite Homeostasis Is a General Rule

A key point is that the results for human muscles are in no way unusual. Similar data for the adenylates, phosphagen, P_i, and H^+ arise from studies of a wide assortment of animals (4–13) as well as other human studies (6). These include invertebrates (7), fishes and other ectothermic vertebrates (8–10), mammals, and birds (see ref. 5). What is more, some of these studies have also analyzed many of the intermediates in specific ATP supply pathways, such as glycolysis (8–12), the Krebs cycle (13), amino acid metabolism, and the β-oxidation pathway of free fatty acid catabolism (see ref. 10 and references therein); here too, changes in pathway intermediates are modest (0.5- to 3-fold) despite large (from severalfold, up to and over 100-fold) changes in pathway fluxes that are simultaneously sustained by the working tissue.

The implications emerging from such studies are (*i*) that ATP is almost perfectly homeostatic under most conditions (except under very extreme fatigue conditions) and (*ii*) that other intermediates in pathways of ATP supply or ATP demand are stabilized within

Abbreviations: MRS, magnetic resonance spectroscopy; PCr, phosphocreatine; CPK, creatine phosphokinase; Mb, myoglobin.

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less rigorously controlled concentration ranges. In one of our earlier analyses (14), the latter condition was described as "relatively" homeostatic because the percent changes in concentrations of intermediates are far less than the percent changes in metabolic rates with which they correlate. For convenience, we shall refer to the homeostasis of substrate concentration, [*s*], in the face of large changes in cell work and cell metabolism, as the [*s*] stability paradox, for which there are several explanations already advanced.

Traditional or Model I Explanations of the [*s*] Stability Paradox

A cursory examination of the literature indicates that currently advanced explanations for metabolite homeostasis at any given step in metabolism depend on the kind of enzyme involved. For simple enzymes obeying Michaelis–Menten kinetics, *in vivo* operation is assumed to be under near-equilibrium conditions with very high catalytic capacities assuring sensitive "high gain" responses to small changes in [substrate]/[product] ratios (see refs. 1, 15, and 16 for literature in this area). Such near-equilibrium function of creatine phosphokinase (CPK) is the accepted explanation for the especially precise regulation of ATP during rate transitions—the traditional ATP "buffering" role of CPK (2). For allosteric enzymes, usually functioning far from equilibrium under *in vivo* conditions, large changes in rate can often be sustained with relatively modest change in key modulators. A quintessential example that fits this pattern is phosphofructokinase (PFK) regulation by several modulators that operate mainly through effects on enzyme–substrate affinity, rather than through changes in maximum reaction velocity. Substrate and product concentrations, however, would be expected to change drastically during large-scale allosteric activation of PFK, because comparable *in vitro* catalytic rates require the enzyme to approach saturation with its substrates (see ref. 10). In liver and other tissues, where the difference between rest and maximally activated metabolism is modest, a widely accepted model used to explain stable concentrations of adenylates (and other intermediates) at varying ATP turnover rates assumes coordinate control by Ca^{2+} of both ATP supply and ATP demand pathways (see ref. 17 and literature therein). These mechanisms, formally similar to other allosteric regulations, apply only to Ca^{2+}-sensitive steps, which represent only a small fraction of all the enzyme-catalyzed reactions in ATP demand and supply pathways. For muscle and heart, these Ca^{2+}-mediated mechanisms in any event seem inadequate to account for the large rate changes observed, and the same may apply for the kidney, which can sustain a very high metabolic scope between ischemic, low-flow states and maximally activated, high-flow states (4, 10). In a third category are enzymes that are regulated by phosphorylation–dephosphorylation or other covalent modifications; when coupled with signal amplification (18), large changes at these specific loci in metabolism can be achieved with modest change in substrate/product concentrations, but again these processes apply to only a modest subset of enzymes in the complex web of pathways that contribute to ATP turnover during cell work. In cases involving covalent modification, the ratio of catalytically active to inactive enzyme is the main parameter being modulated; this is the main reason why change in reaction rate can occur with minimal change in substrate concentrations. We generalized this concept and reasoned (5, 19) that the simplest model to account for widespread metabolite homeostasis assumes regulation of the concentrations of catalytically active enzymes in pathways of both ATP demand and ATP supply (e_o regulation). This would achieve changes in ATP turnover rates proportional to the k_{cat} of the enzymes involved with no required change in substrates or products. Such regulation could be achieved by protein–protein based "on–off" switching between active and inactive forms of enzymes, as in actomyosin ATPase (5), by redox-based "on–off" switching, as in V-type ATPases (20), or by translocation from an inactive to an active intracellular location (essentially isolating enzymes from their target substrates), as in glucose transporters (21).

In short, to explain metabolite homeostasis in varying metabolic states with simultaneous precision and integration of linked sequences of enzyme function, several regulatory models are currently being evaluated by workers in this field (1, 4, 6, 22–29). These include: (*i*) simple feedback and mass action controls (for so-called equilibrium enzymes), (*ii*) allosteric controls (for regulatory enzymes such as phosphofructokinase), (*iii*) models involving the regulation of e_o (the concentration of functional catalytic sites by means of alteration in protein interactions, by change in phosphorylation state, by change in redox state, or by translocation from inactive to an active intracellular location), and additionally, (*iv*) various versions of metabolic control analysis originally introduced over a decade ago (see ref. 30).

Such studies are admittedly, if variably, successful in explaining metabolite homeostasis during changes in work rate [some, like metabolic control analysis, are empirical mathematical models that do not directly address the issue of mechanisms of metabolite homeostasis and, in fact, recognized this as an issue only after our papers began to appear in the literature (31)]. Despite some admitted success of these earlier analyses, for models assuming key regulatory roles for pathway intermediates, the striking homeostasis of most metabolites consistently presents a thorny problem that has not really been acceptably explained: namely, the percent change in putative regulatory intermediate is always less than the percent change in flux required to match the change in ATP turnover rate. Put another way, the kinetic order is usually <1, too low for change in [*s*] to be "driving" the observed flux or metabolic rate changes. Given that this is observed for all categories of enzymes discussed above, it would be a statistical miracle to observe similar [*s*] stability for all of them. Yet a cursory count for pathways of glucose, fat, and amino acid metabolism (5, 10) shows that the percent changes in concentrations of more than 60 substrates and intermediates quantified to date are far less than the percent changes in pathway and enzyme flux rates with which they correlate. The only metabolite that seems to be an exception is oxygen. Even this turns out not to be a real exception, but the research here is so instructive that it is useful to reason our way through the empirical evidence.

Oxygen Delivery Is Fundamental to Metabolic Regulation

A huge literature has developed on how O_2 functions both as a substrate and as a potential regulator of tissue metabolism over varying times of exposure (32–40), and I shall not review this comprehensively at this time. Suffice it to emphasize that over and over again numerous studies have found essentially 1:1 relationships between O_2 delivery and muscle work, in some cases somewhat offset by changes in O_2 extraction. For example, in recent studies using a dog gastrocnemius preparation (26, 33), we found such a relationship between O_2 delivery and work over an 18-fold change in ATP turnover rate. Later, Hogan *et al.* (27) used the same preparation to analyze subtle submaximal work changes. These transitions were sustained with immeasurable change in [PCr], [P_i], and [ATP]; presumably, therefore, the concentrations of other metabolites in participating metabolic pathways were also stable, as in other systems (4, 5, 11). Yet, through these transitions, a 1:1 relationship between change in work and change in O_2 delivery was maintained. Because these kinds of results are qualitatively similar to those found in many other studies, we and many others in the field accept that O_2 plays a key role in regulating change in ATP turnover (5). But how is the O_2 signal transduced within the cell?

Oxygen Signal Transduction in Working Muscle

Unfortunately, the answer to this question remains unclear, and the only mechanisms proposed by traditional studies in this area assume the Krogh cylinder and calculate smooth diffusion gradients within the cell ending in mitochondrial O_2 sinks. So far, this approach has been less than satisfactory because, to unravel the puzzle of how O_2 delivery translates into effects on metabolism within the cell, we

require hard data on intracellular O_2 concentration. The problem is that for most tissues this key parameter remains elusive and unknown. The situation in muscle is more favorable, however. In this tissue, myoglobin (Mb) supplies a direct intracellular detector of $[O_2]$. Mb is a relatively small, monomeric respiratory pigment occurring in heart and mitochondria-rich skeletal muscles at concentrations of <0.5 mM; in muscles of marine mammals such as seals, Mb concentrations reach into the 4–5 mM range. Gene knockout experiments (34, 35) show that mice can survive without Mb (34) but that they can do so only by activating compensating mechanisms such as increasing capillary densities and blood O_2 carrying capacity (35). It is therefore usually assumed that Mb is functionally important under usual physiological conditions. At 37°C, O_2 solubility in physiological solutions is about 1 μM/torr (1 torr = 133 Pa). Because the reaction Mb + $O_2 \rightleftharpoons MbO_2$ is always in equilibrium, with a P_{50} of 3 torr (K_d of about 3 μM), whenever $[O_2]$ is less than saturating for Mb, measures of percent MbO_2 directly estimate intracellular $[O_2]$. Earlier attempts at such measurements with working muscle preparations relied almost exclusively on near infrared spectroscopy (see ref. 36 and references therein). More recently, MRS is being used to take advantage of a histidine-H being ^{1}H MRS "visible" in deoxyMb but being MRS "invisible" in oxyMb. This new technology supplies workers in the field with a noninvasive window on the oxygenation state of muscles in different work and metabolic states, at least for muscles with a high enough Mb to be ^{1}H MRS "visible." When this method was applied to both working human skeletal muscles (37) and to heart (38), the same striking results were reported: essentially stable percent MbO_2 through large changes in work rate. In such tests, as soon as a work load is imposed [even very low intensity exercise, such as unloaded pedaling (37)], percent MbO_2 quickly establishes a new steady state, usually between 40% and 70% saturation, both as a function of time (36) and as a function of tissue work intensity (37, 38). Along with gold-labeling studies showing a random Mb distribution in rat heart and skeletal muscles (S. Shinn and P.W.H., unpublished data), the MRS data imply that percent MbO_2 and intracellular $[O_2]$ both remain relatively constant up to the maximum sustainable aerobic metabolic rate of the tissue (37, 38). As CPK serves to "buffer" ATP concentrations during changes in muscle work, so Mb apparently serves to "buffer" intracellular oxygen concentrations in different metabolic states. Parenthetically, it should be acknowledged that the region of interest in these kinds of MRS studies is large, and the MRS data necessarily are averages of large numbers of fibers. Human muscles, like muscles in other mammals, are formed from mixtures of fiber types, and as work intensity rises for a given muscle mass, there may be changes in recruitment and in the percent contribution of different fiber types. This problem does not arise in studies of heart muscle, which is biochemically rather homogenous (38). Whereas Richardson *et al.* (37) apparently avoided this artifact, this does not seem to be the case in a recent study (39) on an unknown mix of fibers in human calf muscle. Evidence of the problem initially arises from the ^{31}P MRS data, which showed an expected linear decrease in [PCr] as work increased; at maximum aerobic work, [PCr] changed by maximally about 3-fold (39). Because the same [PCr] change occurs when gastrocnemius work rate reaches only 40% of sustained aerobic maximum, but much smaller changes in [PCr] occur in (the mainly slow fibers of) soleus during the same work transition (2), it is probable that the regions of interest in the Mole *et al.* (39) study may have overlapped into muscles rich in slow-twitch fibers, where the change in [PCr] is less for a given level of work than in fast-twitch fibers (2). Otherwise, it would be difficult to understand why their preparation had to be pushed to its maximum work level to achieve the same percent [phosphagen] shifts that we observed at only 40% of aerobic maximum (2). For these reasons, the percent MbO_2 values recorded at different work intensities almost certainly represent different combinations of fiber types. Nevertheless, these studies (39) reported that at about 50% and 80% of sustained aerobic maximum work rate [representing huge ATP turnover rates, equivalent to about 50–80 μmol of ATP $\times$ g^{-1} $\times$ min^{-1} (5)], percent MbO_2 (65–70%) did not change significantly, in agreement with earlier studies (37), whereas at the maximum work rate, a further modest desaturation to about 50% MbO_2 occurred, which is not in full agreement with earlier data (37). Because of the mixed fiber and recruitment problems, we are not surprised by these modestly different results, and, at least tentatively, we consider that the small discrepancies probably arise from artifacts caused by differing metabolic states in different fiber types. Thus, they do not strongly influence our main conclusion that $[O_2]$ is largely homeostatic.

In fact, even if most workers probably would accept that Mb should function to buffer intracellular $[O_2]$, the significance of this has not been fully appreciated. As Carl Honig explained to the author in a discussion in 1987, this may be because of a too enthusiastic acceptance of traditional diffusion models assuming smooth gradients across the capillary–muscle cell threshold all the way to the mitochondrial sinks. Such models (see ref. 39 for an example), which assume complete homogeneity and necessarily ignore the issues of fiber type and recruitment heterogeneity, are not accepted by the Honig group (40). According to Honig *et al.*, the structure of the capillary–muscle system develops steep gradients (and localized high O_2 fluxes) only at the capillary–muscle interface, but very shallow gradients within the muscle cell *per se*, as indeed found by the above later MRS data on percent MbO_2 *in vivo* (36–38). That is why, in one of our earlier reviews (14), we already accepted the MRS data on percent MbO_2 at face value and emphasized that, under normoxic conditions, O_2 is perfectly homeostatic in the sense that its concentration is stable even while its flux to cytochrome oxidase can change by two or more orders of magnitude. In the examples given (1, 37, 39), the concentration of O_2 ranged between 2 and 4 μM during pathway flux changes from about 1 to >80 μmol of ATP $\times$ g^{-1} $\times$ min^{-1} [these high mass specific metabolic rates are attainable because most of the cardiac output during these protocols is available for supporting the work of relatively small muscle masses (see ref. 5)].

To recapitulate, the situation arising from these new studies of O_2 and metabolic regulation can be summarized as follows: First, because of the buffering role of Mb, O_2 concentrations are low (in the P_{50} or K_d range of about 3 μM), and intracellular $[O_2]$ gradients must be quite shallow. The latter point is more fully discussed by the Rochester group (24, 40); one of the most important insights emphasized by these researchers is that the capillary–muscle contact surface area is only a fraction of the surface area of inner mitochondrial membranes and cristae; at steady state, of course, the same net O_2 transfers are occurring at both sites. That is why the highest gradients and highest O_2 fluxes must be at the smaller contact zones (i.e., at capillary–muscle cell interfaces) and why O_2 gradients are necessarily much shallower in the cytosol. Second, the low intracellular $[O_2]$ is powerfully "buffered" by Mb and remains essentially stable throughout large changes in work and metabolic rates. Thus the [*s*] stability paradox (constant [*s*] when flux and hence enzyme–substrate encounter and catalysis rates are increasing) applies to O_2 as well as to other metabolites. Nevertheless, O_2 consumption and O_2 delivery are closely related, suggesting a key role for oxygen in metabolic regulation.

Given that it is O_2 delivery, not intracellular $[O_2]$, that correlates with work rate, the problem we are left with is the issue of how the O_2 signal is transmitted to the machinery of cell metabolism. At this time, we admit that there is no widely accepted answer. When we first recognized this puzzling situation, we proposed a model that postulates an O_2 sensing system presumably located in the cell membrane (or even more distally) and signal transduction pathways or mechanisms for "telling" the cell metabolic machinery when and how potently to respond to changing availability of O_2 (5). However, the nature and even existence of such sensing and signal transducing systems remain to be elucidated. In any event, this and all of the

other above attempts to explain the [*s*] stability paradox are based on diffusion control of change in enzyme–substrate encounter rates. Model II questions this assumption. It takes an entirely different tack and postulates that intracellular circulation, not diffusion, is the main means for bringing ligands and their binding sites together during upwards or downwards transitions in metabolic and tissue work rates.

Model II: Explaining the [*s*] Stability Paradox with Intracellular Structure and Intracellular Perfusion Systems

Conceptually, the major difference between the above traditional approach to metabolic regulation and model II is the emphasis placed on intracellular order and structure. The point of departure for the latter view is that the cell is not a bag of enzymes; instead, it assumes that most metabolic systems operate within an ordered milieu and that important functional consequences arise. Time and space will not allow a detailed review of the evidence for this position. Suffice it to emphasize that it arises from a variety of approaches and that the overall hypothesis is constructed from several different lines of evidence favoring intracellular perfusion and lines of argument not favoring diffusion as the main means for changing the rates at which enzymes and their substrates are brought together. First and most fundamental is the structural argument: ultrastructural, histochemical, and cytochemical studies do not indicate the cell as a static bag of enzymes, but rather a three-dimensional membrane-bound microcosm housing an internal milieu filled with complex organelles, motors, membranes, cables, trabecullae, pumps, and channels. Rather than a static, dead-still solution [as would be required for formal application of laws of diffusion (41)], the internal media of cells are very much "alive" in the sense that movement is the rule of thumb, movement of organelles, of particles, and of cytosol. In large cells, so-called cytoplasmic streaming occurs at rates from <1 to about 80 μm/sec (42, 43). The process is metabolically controlled (44), varies with metabolic state (42), is based on ATP-dependent and ATP-utilizing myosin motors [so-called unconventional myosin isoforms (45)] that can be activated to run on actin filaments (45, 46), and behaves for practical purposes like an intracellular circulation system. What is more, because of the conservative nature of macromolecular structures and functions, we have good reasons for thinking that this, and comparable systems based on kinesin and dynein motors running on microtubules, are widespread and probably characteristic of all cells (46). Additionally, in contrast to what might be expected of a bag of enzymes, over a half-century of research has clearly concluded that many metabolic pathways and their component enzymes are restricted to specific cell compartments, and numerous so-called soluble enzymes (see ref. 47 for a recent study of aldolases) show intracellular binding to specific intracellular sites (48, 49). Order, structure, and circulation are thus the key players in the game, as far as the literature on cell ultrastructure is concerned, and it is not a diffusion-dominated game. Take away the order and the system behavior falls apart; sometimes function is lost completely. A good recent example of this comes from genetic studies of *Drosophila* flight muscle metabolism. Whereas earlier studies had shown that aldolase, glyceraldehyde-3-phosphate dehydrogenase, and α-glycerophosphate dehydrogenase colocalize mainly at Z-discs, Wojtas *et al.* (50) used clever genetic manipulations (that influenced binding but not overall catalytic activities) to show that mislocating these enzyme activities in the cytosol rather than correctly bound to Z-discs would render *Drosophila* flightless. This is a compelling demonstration that enzyme–substrate encounter by simple diffusion mechanisms is inadequate to maintain function, even if all three enzymes are expressed at adequate activities; their three-dimensional organization is part and parcel of *in vivo* regulated function of the pathway.

Second is the argument on macromolecular diffusional constraints. As we might expect from the above (and indeed find), the intracellular mobilities of enzymes and of carrier proteins such as Mb are not equivalent to those in simple aqueous solutions. For example, intracellular diffusibility estimates for Mb in the cytosol range from as low as 1/10 that found in simple solutions (51) to values of about 1/2 (52). Interestingly, the latter MRS study estimated rotational diffusion, whereas Juergens *et al.* (51) estimated translational diffusion and these may change independently (53). Be that as it may, even so-called soluble cytosolic enzymes and other proteins are also apparently highly restricted in their intracellular mobility (51); again, this picture is not easily compatible with the concept of the cell as a bag of easily diffusible enzymes. Order and structure seem to be constraining the intracellular behavior of macromolecules, and their restricted mobilities would not facilitate the kind of enzyme–substrate encounters required for simple solution models of cell function. In contrast, an intracellular perfusion system could easily circumvent these kinds of limitations on bringing enzymes and substrates together.

Third is the argument on metabolite mobility. Because of the complexity of the internal milieu, the translational mobility even of simple molecules may be restricted compared with simple solutions (53), and this is especially true in the mitochondrial matrix (54). A recent study dissected different contributions to limiting mobility of intracellular metabolites. Compared with water, hindrance to translational diffusion in cytoplasm could be attributed to three independent factors—viscosity, binding, and interference from cell solids: (*i*) fluid-phase cytoplasmic viscosity in the fibroblasts used in the study was nearly 30% greater than water; (*ii*) nonspecific, transient binding of small solutes (such as the fluorescent probe used in the study) by intracellular components of low mobility decreased metabolite mobility by about 20%; and (*iii*) translational diffusion of small solutes was hindered 2.5-fold by collisions with cell solids comprising about 15% of isosmotic cell volume. Together, these three factors could account for translational diffusion in cytosol that was decreased to only 27% that observed in water (53). Interestingly, these studies also demonstrated that, during osmotic stress (cell volume increasing to 2 times isosmotic volume, during which the cells sustained a proportional increase in metabolism as part of osmoregulatory costs), the relative translational diffusion coefficient increased by about 6-fold, while the rotational diffusion constant remained constant. Similar insights arise from recent studies of the phosphagen system in vertebrate muscles. Recall that two fundamental assumptions underlie traditional dogma on CPK function in phosphagen-containing cells: (*i*) CPK always operates near equilibrium, and (*ii*) CPK has access to, and reacts with, the total pool (tCr) of PCr and creatine (Cr). Recently, we tested the latter assumption in fish fast-twitch muscle by introducing [^{14}C]Cr into the muscle pool *in vivo* (55). Current model I theory would predict that at steady state, after [^{14}C]Cr administration, the specific activities of PCr and Cr should be the same under essentially all conditions. In contrast, we found that the specific activity of PCr greatly exceeded that of Cr in various metabolic states between rest and recovery from exercise. The data imply that a significant fraction of Cr is not free to rapidly exchange with exogenously added [^{14}C]Cr; releasing of this unlabeled or "missing" Cr on acid extraction accounts for lowered specific activities. Because Cr dominates tCr only in fatigue states, the reduced mobilities implied by these studies correlate with states of lowered metabolic rate. In a follow-up study, ^{1}H MRS was used to further evaluate the *in vivo* behavior of (the methyl triplet of) tCr in human gastrocnemius muscle. We found (56)[†] that the T_2 values for tCr decrease on transition from rest (through a volitional exercise protocol) to ischemic fatigue. In ischemic fatigue, the ATP turnover rate of human calf muscle is severely depressed (5).[†] Because Cr forms the bulk of tCr in ischemic fatigue, its MRS behavior (especially the reduced molecular mobility implied by the

[†]Trump, M. E., Allen, P. S., Gheorghiu, D., Hanstock, C. C. & Hochachka, P. W., Proceedings of the International Society of Magnetic Resonance Meeting, 1997, Vancouver, p. 1337.

reduced T_2 values) is consistent with the earlier ^{14}C results and may explain the mystery of "missing" creatine in the ^{14}C study. The key point is that, just as in the Kao *et al.* fibroblast study (53), the solution behavior of metabolite-sized molecules such as Cr seems to be a function of the metabolic state of the tissue—high molecular mobilities (caused in part by high intracellular circulation rates?) correlating with high metabolic rates. In all of these kinds of studies, order and structure seem to dominate the intracellular behavior of micromolecules such as metabolite intermediates, and serious constraints on diffusion would again not readily facilitate large-scale increases or decreases in enzyme–substrate encounters as required for simple solution models of cells functioning in widely varying activity and metabolic states. Again, these limitations could be easily circumvented with intracellular circulation systems.

Given that enzymes are structurally localized and not free to readily diffuse about and that substrates are also relatively restricted compared with simple solutions, workers in this area (41, 57, 58) consider diffusion by itself to be an inadequate, inefficient, and minimally regulatable means of delivering carbon substrates and O_2 to appropriate enzyme targets in the cell under the variable conditions and rates that are required *in vivo*. Instead, an intracellular circulation or convection system is proposed as an elegantly simple "assist" mechanism providing for the efficient bringing together of substrates (including O_2) and enzymes under varying metabolic conditions. The main evidence for this concept is indirect and comes from studies showing cytoplasmic steaming at velocities far exceeding those to be reasonably expected from diffusion alone, especially in the absence of steep [metabolite] and [O_2] gradients. As mentioned above, such intracellular movement is known to be [possibly Ca^{2+} (44)] regulated and to be based on two kinds of molecular motors: myosin motors traveling on actin filaments and kinesin or dynein motors traveling on tubulin tracks (59, 60). Even organelles such as mitochondria display metabolically regulated movement in cells; actin and tubulins can both be used as tracks for moving mitochondria, but questions of where and how such motors interact with (and are localized on) the outer mitochondrial membrane are not yet fully resolved (61). Nevertheless, mitochondrial-bound myosins are clearly required for directional movements of mitochondria (62), and a recent study showed that depolymerization of F-actin causes a large (5-fold!) decrease in the velocity of mitochondrial movement (63), presumably coincident with a large drop in O_2 consumption caused by the same kind of manipulation (64). Except for a few recent analyses (41, 57, 58), the metabolic implications of such intracellular convection systems have been completely overlooked (or ignored). However, the idea of intracellular convection as a means for increasing enzyme–substrate encounter rates with increasing tissue work is quite compelling. Not only is the rate of cytoplasmic streaming variable [over at least an 80-fold or more range (42, 43) as would be required *in vivo*], in several cell systems (43, 65) there is evidence for a direct relationship between cell work and cytoplasmic streaming rates; and, in a plant cell model, a linear relationship exists between the myosin motor velocity and the force against which it must operate (66). The ^1H MRS studies mentioned above (56)† show that low metabolic rate states (such as ischemic fatigue) correlate with low molecular mobilities of key metabolites such as Cr (consistent with times of low intracellular circulation rates). Using fibroblasts and osmotic stress, the studies of Kao *et al.* (53) similarily show that increasing metabolic rate correlates with increasing molecular translational mobilities (which, again, could be consistent with increased intracellular circulation rates). Thus, we already have some good reasons for anticipating that changes in intracellular convection correlate with changes in cell metabolic rate, although more studies along these lines are clearly desirable.

From the point of view of the current paper, the key advantage of this model is that it easily explains how enzymes and substrates can be brought together and how reaction rates can occur at widely varying rates with minimal change in substrate concentrations; i.e., it easily explains the [*s*] stability paradox of pathway substrates and intermediates, including O_2. As in the perfusion of tissues such as muscle mentioned above, the rate of intracellular metabolism by this model is a product of intracellular perfusion rate: the greater the intracellular perfusion rate the greater the metabolic rate with no concomitant change in substrate concentrations required—a coarse control principle, long and well appreciated by physiologists as the Fick principle. To be sure, this need not rule out other control mechanisms, the kinds that have so far absorbed much of metabolic research; it merely puts them into a different physiological context.

For O_2 transport, this view places Mb function into an entirely different perspective as well, where the fundamental purpose of an intracellular Mb may be to equalize [O_2] everywhere in the cytosol. Functionally, this would ensure that intracellular convection would always be delivering similar amounts of O_2 per unit volume of cytosol to cytochrome oxidases. This model would predict that Mb knockout mice would either be seriously disturbed [as indeed frequently noted (34)], or through ontogeny would develop compensatory mechanisms [as indeed is also observed in those mice that survive Mb gene deletion (35)]. However, from this new point of view, the "buffering" function of Mb, the main function of a half O_2 saturated, randomly distributed Mb, is to ensure a similar [O_2] everywhere in the cytosol (and simultaneously to minimize or even destroy intracellular [O_2] gradients). While this model is consistent with the minimal intracellular [O_2] gradients in muscle cells proposed by the Honig, Connett, and Gayeski work (40), it takes on quite a different meaning. Finally, the concept of an intracellular perfusion system supplies purpose and meaning to intracellular movements (motor-driven or otherwise induced cytoplasmic streaming), which, until this point in time, have been pretty well ignored by traditional metabolic studies.

Diffusion of course is a limited solution to limited problems. Earlier (5), I pointed out that, in the up-regulation of metabolic capacities of skeletal and heart muscles [for example, in organisms such as the hummingbird (67)], the higher the O_2 fluxes required, the shorter the diffusion distances and the less and less dependent on diffusion muscle metabolic organization seems to become. Of course, these same adaptations mean that the higher the fluxes required, the shorter the intracellular perfusion distances. The flight muscles of insects might represent these phenomena close to their limit, with tracheal-supplied mitochondria and myofilaments being packed so tightly together that there is hardly any room left for (and hardly any need for) any intracellular perfusion systems (see refs. 5 and 67 and references therein). Adaptations in the same direction seem to be evident in recent Mb gene knockout studies, which show that some Mb-free mice survive the deletion, apparently because of compensatory mechanisms such as increased capillary densities (35). Although heart muscle cell diameters were not recorded (35), in similar CPK knockout protocols, ontogenetic adjustments led to smaller muscle cells (see ref. 5). Functionally, these adjustments would mean minimizing diffusion and perfusion distances, as in hummingbird and insect flight muscles. To this point, physiologists have generally agreed that organisms get around diffusion limitation problems of O_2 transport by relying on convection systems: ventilation at the lungs and circulation to the tissues, interspersed with diffusion-based steps along the way. The concept of intracellular convection modifies our overall view to include an intracellular component to the chain of convective and diffusive steps in the overall path of O_2 from air to mitochondria (68–70).

In considering the concept of intracellular convection, early pioneers in this field may be prone to over-enthusiastic pressing of their case; this is understandable because it seems to explain so much previously puzzling data so easily (41, 58). Nevertheless, there clearly remain critical functions that are largely or solely diffusion-based, so the understandable over-enthusiasm with which model II proponents minimize the importance of diffusion in energy metabolism puts them at risk of casting away a very useful concept. To

finally assemble a model that can realistically explain a realistic working range of metabolic systems, what seems to be required for the future is an opening up of channels of communication between the above two very differing views of metabolic regulation.

Summary

Acute responses to increases in cell work (to increases in ATP demand) invariably require the activation of ATP supply pathways. The requirements for cell homeostasis would also require that these transitions occur with minimal perturbation of metabolite concentrations, whereas most metabolic regulation models would predict major changes in concentrations of pathway intermediates. Empirically, it is observed that the demands of cell homeostasis prevail; i.e., that during transition from low to high work rates, the concentrations of most substrates in ATP demand and ATP supply pathways are remarkably stable. I term this the [*s*] stability paradox in this paper. Researchers have tried to resolve this paradox while working within two guiding paradigms. The first, model I, looks on the cell as a watery bag of enzymes. Within this framework, several explanations have been advanced to explain the apparent homeostasis of pathway metabolites during small- and large-scale changes in pathway fluxes. While admittedly successful, different mechanisms have to be postulated to account for different kinds of enzymes, and thus different mechanisms have to be postulated for specific loci in metabolic pathways. On balance, we consider it unlikely that all of these different mechanisms would summate to similar [*s*] stabilities observed for more than 60 metabolites at different loci in different metabolic pathways. So, within model I approaches, we consider that the [*s*] paradox remains unresolved.

Model II approaches to metabolic regulation recognize cell structure to be an inherent part of cell function. A subset of these studies places especial emphasis on the fact that the intracellular milieu is not a still, watery solution in which bulk transfer of metabolites occurs mainly by diffusion; instead, it is a three-dimensional structured system in which transport of materials is dominated by intracellular perfusion or convection systems. Current evidence suggests that cytoplasmic streaming (at surprisingly high maximum rates) is controlled by means of controlling molecular motors on actin filaments or on microtubules. Our analysis of the metabolic implications of an intracellular circulation system leads to the concept of intracellular convection as an added and critical means for regulating rates of enzyme–substrate encounter. Increasing enzyme–substrate encounter rates with increasing perfusion rates easily explains changes in pathway fluxes with minimal changes in substrate concentrations. This mechanism for accelerating reaction rates would work equally well at all steps in complex metabolic pathways, no matter what the catalytic and regulatory properties of enzymes might be at these loci in metabolism. Indeed, the ease with which the model II (intracellular convection) model explains the [*s*] stability paradox is one of its most appealing features.

Finally, it may be worth emphasizing that developments in the above two research approaches have been progressing for the last three to four decades, along surprisingly independent trajectories, with minimal communication between the two fields. The usual lack of dialogue between the two research approaches is all the more peculiar when it is pointed out that some of us sometimes work within one paradigm, while at other times we work within the other's constraints. I include myself in this situation; for example, the study by Allen *et al.* (2) illustrates model I approaches, while Hochachka and Mossey (55) clearly illustrate a model II preference. Because both paradigms cannot be right, we consider that it may be time to treat the schizophrenia in these two fields, a process that for certain would require opening up communication channels between them. Whether or not this turns out to be possible remains to be seen; nevertheless, the present paper is part of our ongoing attempt to facilitate this process.

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1. Hochachka, P. W., McClelland G. B., Burness, G. P., Staples, J. F. & Suarez, R. K. (1998) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **120,** 17–26.
2. Allen, P. S., Matheson, G. O., Zhu, G., Gheorgiu, D., Dunlop, R. S., Falconer, T., Stanley, C. & Hochachka, P. W. (1997) *Am. J. Physiol.* **273,** R999–R1007.
3. Hochachka, P. W., Clark, C. M., Holden, J. E., Stanley, C., Ugurbil, K. & Menon, R. S. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1215–1220.
4. Balban, R. S. (1990) *Am. J. Physiol.* **258,** C377–C389.
5. Hochachka, P. W. (1994) *Muscles and Molecular and Metabolic Machines* (CRC, Boca Raton, FL).
6. Hogan, M. C., Richardson, R. S. & Haseler, L. J. (1999) *J. Appl. Physiol.* **86,** 1367–1373.
7. Wegener, G., Bolas, N. M. & Thomas, A. A. G. (1991) *J. Comp. Physiol. B* **161,** 247–256.
8. Dobson, G. P. & Hochachka, P. W. (1987) *J. Exp. Biol.* **129,** 125–140.
9. Hochachka, P. W. & Guppy, M. (1987) *Metabolic Arrest and the Control of Biological Time* (Harvard Univ. Press, Cambridge, MA).
10. Hochachka, P. W. & Somero, G. N. (1984) *Biochemical Adaptation* (Princeton Univ. Press, Princeton).
11. Dobson, G. P., Parkhouse, W. S., Weber, J. M., Stuttard, E., Harman, J., Snow, D. H. & Hochachka, P. W. (1988) *Am. J. Physiol.* **255,** R513–R519.
12. Hochachka, P. W., Bianconcini, M., Parkhouse, W. S. & Dobson, G. P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5764–5768.
13. Rowan, A. N. & Newsholme, E. A. (1979) *Biochem. J.* **178,** 209–216.
14. Hochachka, P. W. & McClelland, G. B. (1997) *J. Exp. Biol.* **200,** 381–386.
15. Betts, D. F. & Srivastava, D. K. (1991) *J. Theor. Biol.* **151,** 155–167.
16. Staples, J. F. & Suarez, R. K. (1997) *J. Exp. Biol.* **200,** 1247–1254.
17. McCormack, J. G. & Denton, R. M. (1990) *Biochim. Biophys. Acta* **1018,** 287–291.
18. Koshland, D. E., Jr. (1998) *Science* **280,** 852–853.
19. Hochachka, P. W. & Matheson, G. O. (1992) *J. Appl. Physiol.* **73,** 570–581.
20. Harvey, W. R. & Wieczoreck, H. (1997) *J. Exp. Biol.* **200,** 203–216.
21. Norris, A. J., Martin, S. S., Harita, T., Nelson, J. G., Vollenweider, P., Gustafson, T. A., Mueckler, M., Rose, D. W. & Olefsky, J. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8401–8406.
22. Atkinson, D. E. (1977) *Cellular Energy Metabolism and Its Regulation* (Academic, New York).
23. Atkinson, D. E. (1990) in *Control of Metabolic Processes*, eds. Cornish-Bowden, A. & Cardenas, M. L. (Plenum, New York), pp. 11–27.
24. Connett, R. J. & Honig, C. R. (1989) *Am. J. Physiol.* **256,** R898–R906.
25. Kushmerick, M. J., Meyer, R. A. & Brown, T. R. (1992) *Am. J. Physiol.* **263,** C598–C606.
26. From, A. H. L., Zimmer, S. D., Michurski, S. P., Mohanakrishnan, P., Ulstad, V. K., Thomas, W. J. & Ugurbil, K. (1990) *Biochemistry* **29,** 3733–3743.
26. Hogan, M. C., Arthur, P. G., Bebout, D. E., Hochachka, P. W. & Wagner, P. D. (1992) *J. Appl. Physiol.* **73,** 728–736.
27. Hogan, M. C., Kurdak, S. S. & Arthur, P. G. (1996) *J. Appl. Physiol.* **80,** 1313–1321.
28. Suarez, R. K., Lighton, J. R., Joos, B., Roberts, S. P. & Harrison, J. F. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 12616–12620.
29. Suarez, R. K., Staples, J. F., Lighton, J. R. & West, T. G. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 7065–7069.
30. Kashiwaya, Y., Sato, K., Tshuchiya, N., Thomas, S., Fell, D. A., Veech, R. L. & Passonneau, J. V. (1994) *J. Biol. Chem.* **269,** 25502–25514.
31. Thomas, S. & Fell, D. A. (1998) *Eur. J. Biochem.* **258,** 956–967.
32. Hochachka, P. W. (1998) *Am. Zool.* **37,** 595–603.
33. Arthur, P. G., Hogan, M. C., Wagner, P. D. & Hochachka, P. W. (1992) *J. Appl. Physiol.* **73,** 737–760.
34. Garry, D. J., Ordway, G. A., Lorenz, J. N., Radford, N. B., Chin, E. R., Grange, R. W., Baseel-Duby, R. & Williams, R. (1999) *Nature (London)* **395,** 905–908.
35. Gödecke, A., Flögel, U., Zanger, K., Ding, Z., Hirchenhain, J., Decking, U. K. M. & Schrader, J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 10495–10500.
36. Tran, T.-K., Sailasuta, N., Kreutzer, U., Hurd, R., Chung, Y., Mole, P., Kuno, S. & Jue, T. (1999) *Am. J. Physiol.* **276,** R1682–R1690.
37. Richardson, R. S., Noyszewski, E. A., Kendrick, K. F., Leigh, J. S. & Wagner, P. D. (1996) *J. Clin. Invest.* **96,** 1916–1926.
38. Jelicks, L. A. & Wittenberg, B. A. (1995) *Biophys. J.* **68,** 2129–2136.
39. Mole, P. A., Chung, Y., Tran, K., Sailasuta, N., Hurd, R. & Jue, T. (1999) *Am. J. Physiol.* **277,** R173–R180.
40. Honig, C. R., Connett, R. J. & Gayeski, T. E. (1992) *Med. Sci. Sports Exercise* **24,** 47–53.
41. Wheatley, D. N. (1998) *BioSystems* **45,** 151–163.
42. Reid, R. J. & Walker, N. A. (1983) *Aust. J. Plant Physiol.* **10,** 373–383.

43. Ryan, P. R. & Walker, N. A. (1994) *J. Exp. Bot.* **45,** 1057–1167.
44. Takagi, S. (1997) *J. Plant Res.* **110,** 299–303.
45. Mermall, V., Post, P. L. & Mooseker, M. S. (1998) *Science* **279,** 527–533.
46. Howard, J. (1998) *Nature (London)* **391,** 239–240.
47. Wang, J., Tolan, D. R. & Pagliaro, L. (1997) *Exp. Cell Res.* **237,** 445–451.
48. Walliman, T., Dolder, M., Schlattner, U., Eder, M., Hornemann, T., O'Gorman, E., Ruck, A. & Brdiczka, D. (1998) *Biofactors* **8,** 229–234.
49. Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. & Eppenberger, H. M. (1992) *Biochem. J.* **281,** 21–40.
50. Wojtas, K., Slepecky, N., von-Kalm, L. & Sullivan, D. (1997) *Mol. Biol. Cell.* **8,** 1665–1675.
51. Juergens, K. D., Peters, T. & Gros, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3829–3833.
52. Wang, D., Kruetzer, U., Chung, Y. & Jue, T. (1997) *Biophys. J.* **73,** 2764–2770.
53. Kao, H. P., Abney, J. R. & Verkman A. S. (1993) *J. Cell Biol.* **120,** 175–184.
54. Scalettar, B. A., Abney, J. R. & Hackenbrock, C. R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8057–8061.
55. Hochachka, P. W. & Mossey, M. K. P. (1998) *Am. J. Physiol.* **274,** R868–R872.
56. Hanstock, C. C., Thompson, R. B., Trump, M. E., Gheorghiu, D., Hochachka, P. W. & Allen, P. S. (1999) *Magn. Reson. Med.* **42,** 421–424.
57. Hochachka, P. W. (1999) *Br. J. Med. Biol. Res.* **12,** 166–176.
58. Wheatley D. N. & Clegg, J. S. (1994) *BioSystems* **32,** 83–92.
59. Langford, G. M. (1995) *Curr. Opin. Cell Biol.* **7,** 82–88.
60. Simon, V. R. & Pon, L. A. (1996) *Experientia* **52,** 1117–1122.
61. Hollenbeck, P. J. (1996) *Front. Biosci.* **1,** d91–d102.
62. Krendel, M., Sgourdas, G. & Bonder, E. M. (1998) *Cell Motil. Cytoskeleton* **40,** 368–378.
63. Boldogh, I., Vojtov, N., Karmon, S. & Pon, L. A. (1998) *J. Cell Biol.* **141,** 1371–1381.
64. Kasuya, Y., Keiji, F., Masaki, M. & Haruma, K. (1994) *J. Biomed. Mater. Res.* **28,** 397–404.
65. Yamaya, M., Fukushima, T., Sekizawa, K., Ohrui, T. & Sasaki, H. (1995) *Respir. Physiol.* **101,** 199–205.
66. Chaen, S., Inoue, J. & Sugi, H. (1995) *J. Exp. Biol.* **198,** 1021–1027.
67. Suarez, R. K. (1992) *Experientia* **48,** 565–570.
68. Schmidt-Nielsen, K. (1979) *Scaling: Why is Animal Size So Important?* (Cambridge Univ. Press, Cambridge, U.K.).
69. Weibel, E. R., Taylor, C. R. & Hoppeler, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10357–10361.
70. Taylor, C. R., Weibel, E. R., Weber, J.-M., Vock, R., Hoppeler, J., Roberts, T. J. & Brichon, G. (1996) *J. Exp. Biol.* **199**, 1643–1649.