Metabolic buffer analysis reveals the simultaneous, independent control of ATP and adenylate energy ratios

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Determining the underlying principles behind biological regulation is important for understanding the principles of life, treating complex diseases and creating de novo synthetic biology [3]. A significant amount of research into this topic has focussed on feedback regulation [3–11], a ubiquitous mechanism in biology that acts via biological actuators, such as regulated enzymes [12,13]. But there is a crucial open challenge to determine the underlying principles for biological regulation beyond feedback [3]. Buffering—the use of reservoirs of molecules to maintain molecular concentrations—is another widespread mechanism for biological regulation [12,13]. Despite its importance, buffering has received considerably less attention than feedback. In recent work, we studied the interaction of feedback and buffering, where we found that synergies between them are often critical for biochemical regulation [12,14]: Feedback regulates the effect of ‘slow’ disturbances while buffering regulates the effect of ‘fast’ disturbances and stabilizes feedback.

However, we do not know all of the underlying principles of buffers and have a limited methodology for quantifying their effects. Our recent findings describe the principles where there is buffering of a single regulated output. While important, they do not fully describe cases where buffers affect multiple outputs, such as in energy metabolism [15]. Also, the familiar treatment for quantifying the effects of buffering is limited to closed systems (with mass and energy isolated from surroundings), whereas in vivo biological systems are fundamentally open systems [3]. Further, the methodology is heavily focused on pH

1. Introduction

Determining the design principles behind biological regulation is important for understanding the principles of life, treating complex diseases and creating de novo synthetic biology [3]. A significant amount of research into this topic has focussed on feedback regulation [3–11], a ubiquitous mechanism in biology that acts via biological actuators, such as regulated enzymes [12,13]. But there is a crucial open challenge to determine the underlying principles for biological regulation beyond feedback [3]. Buffering—the use of reservoirs of molecules to maintain molecular concentrations—is another widespread mechanism for biological regulation [12,13]. Despite its importance, buffering has received considerably less attention than feedback. In recent work, we studied the interaction of feedback and buffering, where we found that synergies between them are often critical for biochemical regulation [12,14]: Feedback regulates the effect of ‘slow’ disturbances while buffering regulates the effect of ‘fast’ disturbances and stabilizes feedback.

However, we do not know all of the underlying principles of buffers and have a limited methodology for quantifying their effects. Our recent findings describe the principles where there is buffering of a single regulated output. While important, they do not fully describe cases where buffers affect multiple outputs, such as in energy metabolism [15]. Also, the familiar treatment for quantifying the effects of buffering is limited to closed systems (with mass and energy isolated from surroundings), whereas in vivo biological systems are fundamentally open systems [3]. Further, the methodology is heavily focused on pH
Buffering, while biological systems are predominantly non-pH buffers. Thus, there is a requirement for novel theory relating buffering to multiple outputs, biochemical kinetics and the quantification of regulation in open systems.

To understand regulatory roles in the case of energy metabolism, we need to know what outputs are regulated by which mechanisms from what types of disturbances [12,16]. Disturbances, such as changing energy demand, cause deviations in the outputs due to a mismatch of ATP demand and supply, while the regulatory mechanisms reduce these deviations. Studies focus on the following key outputs: ATP concentration, which is the cellular energy currency; and the ATP/ADP and ATP/AMP concentration ratios, which are related to the adenylate energy environment of a cell. We need to use three variables to describe the direction of an aircraft; roll, pitch and yaw (see the below figure, [2]). Without all three, we do not have a complete picture of the aircraft’s direction. To describe the adenylate energy environment of a cell, we similarly need to use three variables as there are three molecules involved (ATP, ADP and AMP). The important outputs are ATP, the ATP/ADP ratio, the ATP/AMP ratio and the energy charge (see introduction for descriptions), noting that one is redundant when all are used in combination.

To control the direction of an aircraft or energy environment of a cell, the three outputs all need to be simultaneously controlled. For an aircraft, the rudder (yaw), ailerons (roll) and elevators (pitch) are the regulatory mechanism that carry out the control of each of these outputs. For energy metabolism, the energy environment is controlled by the reaction rates of the metabolic pathways as well as creatine kinase, adenylate kinase and AMP deaminase.

In the below analysis, we first show that these buffers are required to simultaneously and independently control the three coupled outputs. We then quantitatively characterize the buffers to show which regulatory mechanisms regulate which outputs.
deamidase enable the simultaneous control of ATP and the key adenylate energy ratios, whereas feedback on metabolic pathways is fundamentally limited to controlling one of these outputs. This result significantly differs from, but can also explain, the widespread view that cells primarily regulate for a single adenylate energy output. We also quantify the regulatory effects of the phosphagen buffering system to reveal which mechanisms regulate which outputs. We find that AK regulates the ATP/ADP ratio while AMPD regulates the ATP/AMP ratio and the energy charge. We find that creatine kinase is significantly more effective when the ATP/ADP ratio is highly sensitive to changes. The phosphagen system quantification is shown to match with human muscle and mouse adipocyte data, including with AK knockout data. Together, these results illustrate the synergy of feedback and buffering in cellular biology to simultaneously control multiple outputs.

We use two separate but complementary methods in the paper: in §3, we qualitatively study the control of multiple outputs using control theory, and in §§4 and 5, we quantify the regulatory effect of the phosphagen system using novel buffer analysis.

2. Background: buffer analysis for a single output

Buffering, the use of reservoirs of molecules to maintain molecular concentration, is a widespread mechanism for biomolecular regulation. In recent work, we found that the synergy between buffering and feedback is often critical for biochemical regulation [12,14]. In this section, we provide a background on buffering for systems with a single output. We use a minimal model to help explain the quantification metrics we use for buffering, noting that the metrics described in this section apply generally.

Consider the model of a single regulated species that is buffered (figure 1a).

\[
\dot{y} = \frac{p(y)}{\text{production with feedback}} - \frac{r(y)}{\text{removal}} + \frac{g(y, x)}{\text{buffering}} + \frac{d}{\text{disturbance}}
\]

where \(y\) is an output concentration being regulated, \(x\) is the concentration of the buffering species, \(p\) is the production rate of \(y\), \(r\) is the removal rate of \(y\), \(g\) is the net flux through the buffering reactions, and \(d\) is a disturbance to the system. Incorporation of feedback is represented by the \(y\) dependence of production. The nominal steady state (when \(d = 0\)) occurs when production matches degradation \((p(y) = r(y))\) and the buffer is at equilibrium \((g(y, x) = 0)\). An illustrative example with creatine kinase buffering can be found in figure 1b.

To quantify the regulatory effects of systems with feedback and buffering, we use two key measures of regulation: the sensitivity function and the buffer equilibrium ratio [12]. The sensitivity function quantifies the overall regulatory effectiveness produced by all regulatory mechanisms present (e.g. both feedback and buffering), while the buffer equilibrium ratio is a metric specific to buffering. The latter is independent from other forms of regulation and from the disturbance itself (see electronic supplementary material, S1.4 for analysis of a simple example).

\[
B = \frac{\Delta x}{\Delta y}
\]

where \(\Delta y = y - \bar{y}\) and \(\Delta x = x - \bar{x}\) are the deviations of \(y\) and \(x\) from their undisturbed steady states (set points) \(\bar{y}\) and \(\bar{x}\), i.e. when there are no disturbances (see electronic supplementary material, S1.1). Thus, \(B\) represents the degree to which the effects of the disturbance have been absorbed by the buffer versus permeated through to the system output. The metric, which describes a relationship at (quasi) equilibrium, is important for analysis of both temporal dynamics and steady state of a system (see below). For small disturbances, \(B\) can be locally approximated about the system set point (see electronic supplementary material, S1.2). An equivalent approximation is used to define the well-known ‘buffer capacity’ metric for pH regulation in closed systems (see electronic supplementary material, S3), and for steady-state sensitivity analysis as commonly applied in systems biology [8].

If we linearize the simple case in (2.1) and assume that the buffering reactions rapidly reach (quasi) equilibrium then the model can be simplified to (see electronic supplementary material, S1.1)

\[
(1 + B) \Delta y = - \frac{h \Delta y}{\text{feedback}} - \frac{\gamma \Delta y}{\text{removal}} + d
\]

where \(h = -(\partial p/\partial y)\) is the linearized feedback gain and the buffer concentration is determined by \(\Delta x = B \Delta y\).

We can observe in (2.3) that buffering reduces the rate of change of the output \(\Delta y\) by a factor of \((1 + B)\), which attenuates
the effect of ‘fast’ disturbances (figure 1c) [12]. By contrast, buffering in (2.3) does not affect steady state disturbances, where any changes in \( B \) have no effect on (2.3) when \( \Delta y = 0 \). Thus one use of \( B \) is to measure a buffer’s ability to regulate ‘fast’ disturbances [12,14], where large \( B \) improved regulation. Buffering can also stabilize oscillations caused by feedback, such as those from delay in the feedback mechanism [12]. \( B \) also has an important control-theory interpretation in terms of derivative feedback; the net flux through the buffering reactions is \( g = -B \Delta y \) (see electronic supplementary material, S1.3) [12,14]. In technology, derivative feedback is a critical component of PID (proportional + integral + derivative) controllers, a ubiquitous form of feedback controller [16].

It is important to distinguish \( B \) from the familiar thermodynamic equilibrium constant, \( K \); the latter relates (zero-disturbance) steady-state concentrations directly (e.g. \( K = \frac{y}{\bar{x}} \)), while \( B \) relates their (non-zero disturbance) deviations from steady state.

The sensitivity function is a normalized measure of the change in a system output, \( y \) due to a disturbance, \( d \). Again, it is affected by all internal regulatory mechanisms present. The sensitivity function is written as

\[
\phi = \frac{||\Delta y||}{y} / \frac{||d||}{p} \tag{2.4}
\]

where \( ||\Delta y|| \) represents the size of deviation \( \Delta y \) accumulated over time, \( ||d|| \) represents the size of disturbance \( d \) accumulated over time, \( y \) is the set point, and \( p \) is the production rate of \( y \) at the set point. The exact form of \( ||d|| \) and \( ||\Delta y|| \) depends upon the type of disturbance. For example in figure 1c with an oscillating disturbance, both would measure the amplitude of the oscillations.

The sensitivity function (2.4) is closely related to commonly used sensitivity analysis in systems biology for studying steady states [8], noting that the function used here can incorporate temporal dynamics as well as steady-state analysis.

The reader may find it instructive to compare and contrast the analysis of pH buffering with open-system metrics introduced in §1 to familiar metrics commonly used to analyse closed-system, which can be found in electronic supplementary material, S3 (noting that this comparison is original to this paper).

3. Buffering can enable the control of multiple outputs

In this section, we ask what concentrations and outputs can feedback and buffering control? This question is well suited to controllability analysis from control theory [16]. Controllability determines the ability for regulatory mechanism inputs (e.g. enzyme activity) to change the system states (concentrations). Determining the actions of regulatory mechanisms contrasts to determining the outputs the mechanism responds to or senses. For example, a pathway enzyme may respond to a change in AMP by acting on ATP production, where controllability characterizes the effect on ATP and not the sensing of AMP.

We first use a minimal model to illustrate general principles of controlling multiple outputs with buffering and feedback. We then apply the general principles to demonstrate the phosphagen system’s role in controlling multiple adenylate energy outputs. Using a controllability approach enables us to study the control of multiple adenylate outputs without also having to analyse the control of other metabolites that are involved in ATP production or usage.

3.1. Control of multiple outputs in a minimal model

To study the role of buffering, we use a modification of the minimal model in (2.1) to include two outputs with buffering on the second output. However, in this section we do not assume that the buffer rapidly reaches equilibrium. Consider (figure 2a)

\[
\begin{align*}
\dot{y}_1 &= \frac{p(y_1, y_2)}{\text{production with feedback}} - r(y_1) \text{ removal with feedback} \\
\dot{y}_2 &= \frac{p(y_1, y_2)}{\text{production with feedback}} + r(y_1) + g(y_2, x) \text{ buffering removal} \\
\dot{x} &= -g(y_2, x), \text{ buffering}
\end{align*} \tag{3.1}
\]

where \( y_1 \) and \( y_2 \) are concentrations of two output species being regulated, \( x \) is the concentration of the buffering species, \( p \) is the production rate of \( y_1 \), \( r \) is the removal rate of \( y_1 \), and \( g \) is the net flux through the buffering reactions.

We next show that if there is only one regulatory mechanism input (feedback) then only one output concentration can be independently controlled. However, if there is an extra regulatory mechanism input (i.e. buffering) then both outputs concentrations can be independently controlled. Now

\[
\dot{y}_1 + \dot{y}_2 = \frac{g}{\text{buffering}}. \tag{3.2}
\]

It is possible for feedback on production to control the concentration of \( y_1 \). However, without buffering (\( g = 0 \)) we have \( \dot{y}_1 + \dot{y}_2 = 0 \), which results in constant \( y_1 + y_2 = D \) [23,24].
This implies that if the concentration of $y_1$ is controlled to any particular concentration, then $y_2 = D - y_1$ is also determined and thus $y_2$ is dependent upon $y_1$ or vice versa (figure 2c). Therefore, without buffering then feedback cannot independently control the concentrations of $y_1$ and $y_2$. The total $y_1 + y_2$ is required to be controlled in order to simultaneously and independently control both $y_1$ and $y_2$, or combinations of the two e.g. $y_1$ and $y_2/y_1$. Buffering can control $y_1 + y_2$ and thus enable the concentrations of $y_1$ and $y_2$ to be independently controlled via the net buffering flux $g$ (figure 2c). A representation of the controllability can be seen in figure 2b.

We can note that the buffer in model (3.1) (figure 2) can take the role described in this section, but it can also attenuate fast disturbances that act on $y_2$, as described in §2. Also, although not the focus of this paper, the above regulatory principles are relevant for biological systems other than metabolism, such as signalling and gene regulatory networks.

3.2. The phosphagen system

We next apply these principles to illustrate the role of the phosphagen system [17] in controlling ATP, ADP and AMP. This system is represented by the buffering reactions

$$ATP + Cr \leftrightarrow pCr + ADP \quad \text{(creatinine kinase)}$$

$$ATP + AMP \leftrightarrow 2ADP \quad \text{(adenylate kinase)}$$

$$AMP \rightarrow IMP + NH_4 \quad \text{(AMP deaminase)},$$

where ATP, ADP and AMP are the adenine nucleotides used for energy, pCr is creatine phosphate, Cr is creatine and IMP is inosine monophosphate. IMP can be regenerated to form AMP in separate reactions. The right-hand side of (3.3) and the left-hand side of (3.5) can also include $H^+$, but we do not include them here both for simplicity and so that we can later use standard definitions of equilibrium constant $K$.

We next show that, as above, if there is only one regulatory mechanism input (feedback) then only one adenylate output can be independently controlled. However, if there are two extra regulatory mechanism inputs (i.e. AK and AMPD buffering) then three adenylate outputs can be independently controlled. To study controllability in the phosphagen system, we first look at the effect of adenylate kinase without AMP deaminase. We then use analysis of this subsystem to study controllability of the whole system with AMPD—a form of mathematical induction.

To determine the effect of the adenylate kinase (without AMPD) on controllability, we study the ATP and ADP concentrations and incorporate metabolic pathways that are controlled by various forms of feedback (see figure 3a and electronic supplementary material, S2 for analysis). We ignore any reactions that cannot be regenerated without AK (e.g. ATP → AMP). This case is very similar to the above minimal model if we set $y_1 = ATP$ and $y_2 = ADP$. As above, feedback on metabolic pathways can control the concentration of ATP, but ATP+ADP is constant without the buffer AK and so feedback alone cannot independently control both ATP and ADP. Similarly, creatine kinase also has no ability to change the total ATP+ADP (see electronic supplementary material, S2).

Adenylate kinase ensures system controllability by varying the total ATP+ADP (see figure 3c for graphical representation). This controllability enables the independent control of ATP and ADP, or combinations such as ATP and ATP/ADP (also see electronic supplementary material, S2 for equivalent analysis of polyphosphate kinase).

While AK enables independent control of two outputs, to study the control of ATP, ADP and AMP we also need to incorporate AMPD and regeneration (see figure 3b and electronic supplementary material, S2 for analysis). We ignore any processes that cannot be regenerated via resynthesis of IMP. If necessary, other regeneration reactions can be incorporated in the models as disturbances, rather than regulatory mechanisms, without changing the results e.g. production of AMP from the degradation of RNA. In this model, feedback and AK can control ATP and ADP, but without AMP deaminase ATP+ADP+AMP is constant and thus unable to be controlled. Similar to above, AMP deaminase can vary the total ATP+ADP+AMP and thus can enable the independent control of ATP, ADP and AMP or various combinations.

Thus we can observe that adenylate kinase and AMP deaminase enable the simultaneous, independent control of ATP, ADP and AMP, which is equivalent to enabling the simultaneous, independent control of ATP, ATP/ADP and ATP/AMP (see electronic supplementary material, S2). By contrast, the system with feedback on metabolic pathways but without buffering is not controllable and is fundamentally limited to only controlling one of these outputs.

We can also take an alternative approach to the one used here and treat AK buffering as part of the metabolic system rather than as a control mechanism. For this case, if we ignore AMPD then we have the conserved total ATP+ADP+AMP = $M$ along with the AK equilibrium constant $K_a = [ATP][AMP]/[ADP]^2$, which results in three outputs and

$$\text{(a) Buffering schematic for adenylate kinase (AK) and creatine kinase}$$

$$\text{and feedback on metabolic pathways. AK and AMPD may control other outputs than those illustrated, where only those necessary (nec.) for controllability are shown.}$$

Figure 3. (a) Buffering schematic for adenylate kinase (AK) and creatine kinase—modified from figure 2a (see (A1) for reactions). (b) Buffering schematic for AMP deaminase and regeneration (see (A2) for reactions). (c) Representation of the controllability for the phosphagen system and feedback on metabolic pathways. AK and AMPD may control other outputs than those illustrated, where only those necessary (nec.) for controllability are shown.
two dependencies. Thus, we can observe that feedback is limited to controlling one independent output. However, without treating the buffer as a control mechanism then we do not observe the role of the adenylate kinase in independently controlling multiple outputs.

4. Quantifying buffering

While the controllability analysis in the last section shows what the regulatory mechanisms can and cannot act on, it provides a qualitative result and does not show which outputs particular mechanisms are regulating, or by how much. For this question, we need to quantify the regulatory effects of energy buffers. In this section, we quantify the effects of creatine kinase and adenylate kinase buffering by determining the buffer equilibrium ratio of each. The reader may recall that this ratio is specific to the individual buffer and can measure each buffer’s ability to regulate ‘fast’ disturbances. We will see in §5 that the buffer equilibrium ratio of adenylate kinase is also important for quantifying the regulation of multiple energy outputs.

4.1. Creatine kinase

We first determine the buffer equilibrium ratio for creatine kinase buffering. We find that creatine kinase is most effective at high K and high ATP/ADP ratios (or low K and low ATP/ADP ratios). We also show that the effectiveness of creatine kinase is reduced in human muscles by adenylate kinase. However, this is offset by the boost to the effectiveness of creatine kinase from varying equilibrium constant K (via changing pH). We show that the theory matches experimental data for human muscle during and after exercise.

The buffer equilibrium ratio for the creatine kinase reaction described in equation (3.3) is

\[ B_c = \frac{\Delta x_c}{\Delta y} = \frac{\Delta [pCr]}{\Delta [ATP]} \]

If we initially assume that the equilibrium constant \( K_c \) and other parameters are fixed then (see electronic supplementary material, S4.2)

\[ B_c' = \frac{C}{D(K_c + (1-K_c)(ATP/D))^2} \]  

(4.1)

where \( x_c = [pCr] \), \( y = [ATP] \), \( C = [pCr] + [Cr] \), \( D = [ATP] + [ADP] \), the equilibrium constant is \( K_c = [ATP][Cr]/([pCr][ADP]) \), and C, D and \( K_c \) are the fixed parameters. We can observe that the buffer equilibrium ratio \( B_c' \) in (4.1) increases linearly with \( C/D \), but the relationship between \( B_c' \) and ATP/D is more complicated.

In figure 4(a), we can see that there are two regions with large \( B_{c} \), and consequently where pCr is most effective at regulating ‘fast’ disturbances. These regions occur where \( K_c \) is small together with large ATP/ADP, and where \( K_c \) is large together with large ATP/ADP, noting that ATP/ADP is large when ATP/D is close to 1. Outside of these regions \( B_{c} \) is significantly smaller and consequently the buffer is less effective.

In calculating \( B_c \), we also need to take the varying equilibrium constant \( K_c \) and \( D = ATP + ADP \) into account. For example, during exercise the rate of ATP decreases intracellular pH, which increases the value of \( K_c \). Similarly, parameter \( D \) decreases as ATP levels decrease due to adenylate kinase converting ADP to AMP. For varying \( K_c \) and \( D \), the buffer equilibrium ratio of pCr is (see electronic supplementary material, S4.2)

\[ B_c = B_c' \left(1 - \frac{[ATP]}{[ATP]} \frac{\Delta K_c}{K_c} \frac{\Delta ATP}{k_{pH}} \frac{\Delta ATP}{D} \right) \]

where \( B_{c}' \) is given in (4.1), \( B_c = \Delta D / \Delta ATP \) is the rate of change of \( D = [ATP] + [ADP] \) with respect to ATP (also see §4.2), \( \Delta K_c / \Delta ATP \) is the rate of change of equilibrium constant \( K_c \), with respect to ATP, and total creatine \( C = Cr + pCr \) in (4.1) is assumed constant.

Under high energy demand situations like exercise, \( K_c \) typically increases when ATP drops (via decreasing pH), which shifts reaction (3.3) to the left back towards ATP and increases the counteracting effect of buffering (\( B_c \)). By contrast, AK removes more ADP when ATP decreases, which shifts reaction (3.3) to the right away from ATP and reduces the counteracting effect of buffering (\( B_c \)).

We can compare the above theory to experimental data from literature, where figure 5a shows values of buffer equilibrium ratio \( B_c \) for human muscles during and after exercise (see electronic supplementary material, S4.2 for calculation methods). It can be seen that the theoretical values of \( B_c \) without AK or varying \( K_c \) are not consistent with experimental values, but match with experimental values when these are included (also see electronic supplementary material, S4.2).

We can observe that the experimental value of \( B_c \) for CK ranges from 7.3 to 21. The metric 1/(1 + \( B_c \)) can act as a proxy for the attenuation of fast disturbances by CK (see electronic

![Figure 4. Buffer equilibrium ratios for (a) CK and (b) AK with fixed parameters, which quantify their buffering effects under different conditions. CK has normalization C/D = 1 where C = [pCr] + [Cr] and D = [ATP] + [ADP].](image-url)
If we had instead set ADP as the buffer then the output as
\[ \text{ATP} \] and the buffer or reservoir as
\[ y \]
was (with fixed and varying parameters). A related result for the alternate purpose of determining changes in AMP to changes in ATP can be found in [8,25]—changes in AMP correspond to changes in ATP + ADP if A is fixed (see above) but not if A is varying (see below).

We can observe in figure 4b that buffer equilibrium ratio \( B_i \) is higher for higher \( K_o \) values. However, \( B_i \) is not large (compared to creatine kinase) at high ATP/A and \( K_o \) with a maximum at or near one. Although not shown in the figure, \( B_i \) can become large at very low energy charges where AMP > ATP (see electronic supplementary material, S4.3).

We also need to take AMPD and corresponding regeneration into account by incorporating varying \( A \). For this parameter-varying case, the buffer equilibrium ratio of AK is (see electronic supplementary material, S4.3)
\[
B_i' = 1 + \frac{[\text{ATP}]}{\frac{[\text{AMP}]}{2K_o[\text{ADP}]} + R_m,}
\]
where \( R_m = \frac{\text{AMP}}{\text{ADP}} \) and the adenine nucleotide total \( A = \text{AMP} + \text{ADP} + \text{AMP} \) is a fixed parameter. Equation (4.4) can also be written in a more complicated form as an explicit function of two independent inputs \( K_o \) and ATP/A (see electronic supplementary material, S4.3). A related result is (also see electronic supplementary material, S4.3). It can be seen that the theoretical values of \( B_i \) from (3.4) are not consistent with experimental values when AMPD is not incorporated, but match with experimental values when it is (also see electronic supplementary material, S4.3). It can be noted that the adipocyte data also requires the assumption that \( K \) varies. The effect of AMPD on AK can be seen in figure 5b to be much larger in muscle than in adipocytes, but that the buffer equilibrium ratio is just below one in both cases.

Figure 5b shows that the AK buffer equilibrium ratio \( B_i \) is slightly less than one in human muscle during/after exercise, while the pCr ratio is \( B_i = 7.3 - 21 \) during/after exercise. As the total buffer equilibrium ratio for ATP is \( B_{tot} = B_i + B_a \) [12], we can observe that AK is much less important than pCr in regulating for ‘fast’ disturbances.

5. Quantifying ratio regulation

In this section, we quantify the overall effect of the phosphagen system to regulate key energy outputs: the ATP/ADP ratio, the ATP/AMP ratio and the energy charge. We find that AK regulates the ATP/ADP ratio while AMPD regulates the ATP/AMP ratio and the energy charge. We also find that AK worsens regulation of the ATP/AMP ratio and the energy charge, but this tradeoff is compensated for by AMPD. The reader may recall that the ATP/ADP and ATP/AMP concentration ratios are related to Gibbs free energy, i.e. the ability of the cell to do work, while the

\[
\text{Buffer equilibrium ratio for adenylate kinase is}
\]
\[
B_o = \frac{\text{ATP} + 2\text{ADP}}{\text{ATP} + 2\text{ADP}}.
\]

If we initially assume that the equilibrium constant \( K \) and other parameters are fixed then (see electronic supplementary material, S4.3)
\[
B_o' = \frac{K_o\text{AMP} + 2K_o\text{ADP}}{\text{ATP} + 2K_o\text{ADP}},
\]

where \( K_o = [\text{AMP}]/[\text{ADP}]^2 \) and the adenine nucleotide total \( A = \text{AMP} + \text{ADP} + \text{AMP} \) is a fixed parameter. Equation (4.4) can also be written in a more complicated form as an explicit function of two independent inputs \( K_o \) and ATP/A (see electronic supplementary material, S4.3). A related result for the alternate purpose of determining changes in AMP to changes in ATP can be found in [8,25]—changes in AMP correspond to changes in ATP + ADP if A is fixed (see above) but not if A is varying (see below).

We can observe in figure 4b that buffer equilibrium ratio \( B_i \) is higher for higher \( K_o \) values. However, \( B_i \) is not large (compared to creatine kinase) at high ATP/A and \( K_o \) with a maximum at or near one. Although not shown in the figure, \( B_i \) can become large at very low energy charges where AMP > ATP (see electronic supplementary material, S4.3).

We also need to take AMPD and corresponding regeneration into account by incorporating varying \( A \). For this parameter-varying case, the buffer equilibrium ratio of AK is (see electronic supplementary material, S4.3)
\[
B_i = B_i'(1 + \frac{[\text{ATP}]}{\frac{[\text{AMP}]}{2K_o[\text{ADP}]} + R_m,}
\]
where \( R_m = \frac{\text{AMP}}{\text{ADP}} \) is the rate of change of \( A \) with respect to ATP due the removal of AMP by AMP deaminase and the corresponding AMP regeneration (see electronic supplementary material, S4.3).

AMPD removes AMP as ATP decreases, which shifts reaction (3.4) to the left back towards ATP and increases the counteracting effect of buffering (1B).

We can compare the theoretical buffer equilibrium ratios to human muscle and mouse adipocyte experimental data. Figure 5b shows values of theoretical and experimental \( B_i \). It can be seen that the theoretical values of \( B_i \) from (3.4) are not consistent with experimental values when AMPD is not incorporated, but match with experimental values when it is (also see electronic supplementary material, S4.3). It can be noted that the adipocyte data also requires the assumption that \( K \) varies. The effect of AMPD on AK can be seen in figure 5b to be much larger in muscle than in adipocytes, but that the buffer equilibrium ratio is just below one in both cases.

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adenylate energy charge \((\text{ATP} + 0.5\text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})\) is an index used to measure the overall energy status of biological cells [15]. We also show that the theory matches experimental data for human muscle and mouse adipocytes.

To quantify the regulation of the energy ratios, we determine the sensitivity of the energy ratios to changes in ATP. Determining these sensitivity functions enables a simple means of translating sensitivity function (2.4) for ATP into sensitivity functions for the energy ratios (see electronic supplementary material, S5.5), as well as being important in its own right, e.g. it is analogous to relating the charge (ATP) and voltage (ratios) of a battery. We use ATP as a reference metric as the regulation metrics are most naturally written in terms of ATP concentrations, e.g. disturbances are most naturally represented in terms of ATP demand or supply. We assume that AK is at (quasi) equilibrium and that AMPD and its corresponding regeneration are the primary means of interconversion between AMP and IMP.

The sensitivity of the ATP/ADP ratio, ATP/AMP ratio and energy charge to changes in ATP are (see electronic supplementary material, S5.1)

\[
\begin{align*}
\Delta \% \frac{\text{ATP}}{\text{ADP}} &= \left[ \frac{\Delta \% \text{ATP}}{\text{ADP}} \right] = 1 + \left( 1 - \frac{B_0}{\text{AK}} \right) \frac{\text{ATP}}{\text{ADP}} \\
\Delta \% \frac{\text{ATP}}{\text{AMP}} &= \left[ \frac{\Delta \% \text{ATP}}{\text{AMP}} \right] = 1 + \left( B_0 - \frac{R_m}{\text{AMPD}} \right) \frac{\text{ATP}}{\text{AMP}} \\
\Delta \% E_c/\Delta \% \text{ATP} &= \left[ \frac{\Delta \% E_c}{\Delta \% \text{ATP}} \right] = \left( 1 + \frac{B_0}{\text{AK}} - \frac{2E_cR_m}{\text{AMPD}} \right) \frac{1}{2E_c} \frac{\text{ATP}}{A},
\end{align*}
\]

where \(\Delta \% \text{ATP} = \Delta \text{ATP}/\text{ATP}\) is the percentage change in [ATP], \(\Delta \% [\text{ATP/ADP}] = \Delta \text{ATP}/\text{ADP} / \text{[ATP/ADP]}\), \(\Delta \% E_c = \Delta E_c/E_c\) and \(\Delta \% [\text{ATP/AMP}] = \Delta \text{ATP}/\text{AMP} / \text{[ATP/AMP]}\) are the percentage change in the respective ratios, \(B_0\) is defined in (4.3), \(R_m\) is defined in (4.5), \(A\) is defined below (4.4) and \(E_c = (\text{ATP} + 0.5\text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})\).

Equation (5.1) is plotted in figure 6 and shows that at high ATP/ADP or ATP/AMP ratio then small changes in ATP can result in large changes in both the ATP/ADP and ATP/AMP ratios, respectively (first reported in [26]). This effect occurs as small relative changes in ATP cause large relative changes in ADP and AMP. The energy charge does not suffer from the same sensitivity effects as the other ratios (figure 6).

Equation (5.1) and figure 6 show that AK reduces the sensitivity of the ATP/ADP ratio to changes in ATP, reaching a minimum when \(B_0 = 1\). AK also creates a tradeoff as increasing \(B_0\) worsens the regulation of the ATP/AMP ratio. However, AMPD regulates the ATP/AMP ratio by lessening this sensitivity effect, reaching a minimum when \(R_m = R_{m0}\). The regulation of the energy charge is also worsened by AK and improved by AMPD. It can be noted that the sensitivity functions in (5.1) are not a function of creatine kinase, which through \(K_c = \text{[ATP]}[\text{Cr}] / ([\text{pCr}] [\text{ADP}])\) sets the sensitivity between pCr/Cr and ATP/ADP, rather than that between ATP and ATP/ADP.

This analysis effectively quantifies the regulation of the energy ratios into two forms. First, improving ATP regulation can be observed to improve regulation of the energy ratios, as reduced deviations in the former will reduce deviations in the latter (or vice versa). Through this general means, it is possible for improved regulation of ATP from feedback and pCr to also improve the regulation of the energy ratios (or vice versa). However, due to the sensitivity of the ratios under some conditions, ATP may be well regulated while the energy ratios are poorly regulated. In the second form of regulation, AK and AMPD can reduce the sensitivity of the individual energy ratios to changes in ATP, enabling simultaneous effective regulation of ATP and the ratios. These observations are consistent with §3, where AK’s and AMPD’s abilities to enable simultaneous, independent control of multiple outputs also enables simultaneous, tailored regulation of the different energy ratios. Interestingly, while pCr regulates ‘fast’ disturbances, AK and AMPD reduce the sensitivity of the energy ratios to any deviation in ATP. Thus AK and AMPD help regulate both ‘fast’ and ‘slow’ disturbances.

![Figure 6. Sensitivity of adenylate energy ratios to changes in ATP (as defined in (5.1)). These plots show the regulatory effect of AK (via \(B_0\)) and AMPD (via \(R_m\)) on different outputs.](image-url)
The results in figure 7 match experimental data for human muscle and mouse adipocytes. The inferred regulation of the ATP/ADP ratio by AK is supported experimentally by AK knockout data, which showed human muscle data and F shows mouse adipocyte data (see appendix).

**Figure 7.** (a) Comparison of experimental and theoretical sensitivity of ATP/ADP (left), ATP/AMP (centre) and the energy charge (right) to changes in ATP. (b) Sensitivity reduction of ATP/ADP by AK (left), ATP/AMP by AMP deam. (centre) and the energy charge by AMP deam. (right). A–D show human muscle data and F shows mouse adipocyte data (see appendix).

We can see in figure 7a that the sensitivity functions match experimental data for human muscle and mouse adipocytes. The results in figure 7b show that on average AK reduces the ATP/ADP sensitivity by $3.9/4.5$ (muscle/adipose), AMPD reduces the ATP/AMP sensitivity by $12.8/12.8$ (muscle/adipose) and AMPD reduces the energy charge sensitivity by $6.1/5.4$ (muscle/adipose).

The inferred regulation of the ATP/ADP ratio by AK is supported experimentally by AK knockout data, which resulted in a significant accumulation in ADP and worsening of the ATP/ADP ratio in skeletal muscle [21].

### 6. Discussion

The study of the regulatory roles of the phosphagen system illustrates an important general principle—buffering can enable the simultaneous, independent control of multiple coupled outputs. This role is in addition to its known role. Different buffers can also carry out different roles. In the phosphagen system, AMPD and AK carry out the latter roles. In AK’s case, its ability to enable simultaneous control occurs due to its production and removal of AMP (and thus ATP+ADP), while its lesser second role occurs due to the regeneration of ATP. In an interesting difference between the roles, CK regulates ‘fast’ disturbances while AK and AMPD regulate both ‘fast’ and ‘slow’ disturbances.

How important is enabling simultaneous, independent control? In the case of energy metabolism, there are multiple outputs and their regulation that are widely regarded as biologically important (see §1). The coupling between these outputs implies that simultaneous and independent control is fundamental to this regulation of multiple outputs. Without simultaneous, independent control, the regulation of one output can interfere with the regulation of another, e.g. AK without AMPD worsens the regulation of ATP/AMP and the energy charge. Also, due to the sensitivity of ratio outputs, it can be difficult to regulate a ratio on its own without independent control of both outputs in the ratio. For example, ATP/ADP is typically highly sensitive to changes in ADP, and so its regulation is more effective via control of ATP and ADP (or ATP and ATP+ADP), rather than just ATP. Thus the ability for simultaneous, independent control analysed in §3 is a foundation for the regulation quantified in §§4 and 5.

In the buffer analysis presented, we have focussed on regulation (i.e. minimizing deviations about a set point). However, the controllability analysis discussed in §3 is also applicable to control (i.e. changing the set point itself). The ability to simultaneously control different outputs can enable the energy environment to be optimized for different cells or compartments. AK controls the relationship between different ratios via ATP/ADP $= K_a$ADP/AMP, rather than explicitly controlling the ATP/ADP ratio. For this case, the set point of the ratios can be controlled by changing $K_a$, e.g. by varying Mg$^{2+}$. This control is in conjunction with the other regulatory mechanisms to jointly control the ratios involving ATP, ADP and AMP.

The quantification methods in this paper determine sensitivities in terms of a few key set points (e.g. ATP concentration) and parameters (e.g. equilibrium constants). While these set points and parameters are themselves dependent upon a larger system, our approach enables biological insights and methods of quantification without requiring us to model and/or experimentally measure all drivers of these set points. For example, we were able to quantify the phosphagen system from experimental measurements of ATP, ADP, AMP, Cr and pCr in muscles during/after exercise and in adipose tissue.

Interestingly, the analysis methods are approximations that are most accurate for small changes, but had good fits for experimental data from exercise that had large disturbances and large changes in metabolites, e.g. depletion of creatine phosphate.

Throughout this study, we have shown that multiple energy outputs are simultaneously regulated by different buffering and feedback mechanisms in a synergistic fashion. This finding differs from a general view that cells primarily regulate for one energy output, typically the energy charge or a related AMP-based output [15]. However, our findings are still consistent with this view if buffering is ignored as feedback on its own can only simultaneously control one adenylate energy output.

The quantification of metabolic buffers shows that the ATP/ADP and ATP/AMP ratios are highly sensitive to small changes in ATP when they are at high levels (first reported in [26]). It also shows how strongly the phosphagen system regulates these ratios when the ATP/ADP and ATP/AMP ratios are at high levels. Both creatine phosphate and adenylate kinase have large regulatory effects when the ATP/ADP ratio is highly sensitive to changes, while AMP deaminase has a large regulatory effect when the ATP/AMP ratio is highly sensitive to changes.
Interestingly, this observation combined with our recent studies about the interaction of buffering and feedback [12,14] would indicate pCr should also stabilize glycolytic feedback more strongly at high ATP/ADP and thus enable feedback to be more effective under these conditions. We can also observe in figure 7 that while the energy charge (the standard energy output) has the ‘steadiest’ output (figure 7a), it is not necessarily the most strongly regulated (figure 7b). In human muscles, the ATP/AMP ratio is significantly more strongly regulated than the energy charge by AMPD, although they are more comparable in mouse adipocytes.

In control theory, the simultaneous, independent control of multiple coupled outputs is referred to as ‘multivariable control’ (see also box 1), which is a topic that has been extensively studied in non-biological contexts [1] but not in biological ones. In this paper, we provide a foundational case study for multivariable control and cellular biology. As the methods are generic and both buffering and feedback are ubiquitous in biology, we believe that this topic will have significantly wider applicability than the case study provided.

7. Conclusion
In this paper, we used control theory and novel buffer analysis for open systems to study buffering in energy metabolism. This enabled us to both uncover underlying principles in metabolic regulation and to quantify the effects of critical regulatory mechanisms. We showed the importance of adenylate kinase along with AMP deaminase and its regeneration for simultaneous, independent control of ATP and the adenylate energy ratios. We also quantified their effect and showed that AK regulates the ATP/ADP ratio, while AMP deaminase regulates the ATP/AMP ratio and the energy charge. Similarly, we quantified the effect of creatine phosphate, and showed that it is significantly more effective when the ATP/ADP ratio is highly sensitive to changes. The results were shown to be consistent with experimental data for human muscle and mouse adipocytes.

These results reveal a fundamental role of buffering in biological regulation: enabling the simultaneous, independent control of multiple outputs, which adds to its known role of regulating ‘fast’ disturbances and stabilizing feedback. These results also reveal that different buffers carry out different roles—in the phosphagen system, AMP deaminase and adenylate kinase carry out the former role, while creatine phosphate and adenylate kinase (to a much lesser extent) carry out the latter role.

Data accessibility. All data generated or analysed during this study are included in this published article and its electronic supplementary material. Data sources for figures 5 and 7 are A [27], B [28], C [29], D [30], E [31], F [32]. For repeated cases, the first case shows changes during ATP depletion and the second case shows changes during ATP regeneration.

In figure 6, (b) shows sensitivity with $K_a = 1$ and (c) shows sensitivity with $K_a = 1$ and $B_a = 1$.

Simulations of the model (2.1) in figure 1 use constant $p$, $r = g_2$, where $g = 0.1$, and $g = -b_1 y + b_{-1} x$ where $b_1 = 500$, $b_{-1} = 100$ for the buffering case (resulting in $B = 5$). Simulations of the model (3.1) in figure 3 use $p = u_x$, $r = g y$ and $g = 0$, $r - p$, where $u_x$ is a control input (negative unit step) and $g$ is a kinetic constant.

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Appendix A

A.1. Reaction modelled for controllability analysis of the phosphagen system
The reaction used to study controllability with adenylate kinase or creatine kinase are

\[
\begin{align*}
\text{ATP} + \text{AMP} & \rightarrow 2\text{ADP} \\
\text{ADP} & \rightarrow \text{ATP} \\
\text{ATP} & \rightarrow \text{ADP} \\
\text{ATP} & \rightarrow \text{AMP}
\end{align*}
\]

where OP is oxidative phosphorylation. For simplicity, we do not include creatine or creatine phosphate in the CK reaction.

The reaction used to study controllability with AMP deaminase and regeneration are (figure 3b)

\[
\begin{align*}
\text{AMP} & \rightarrow \text{IMP} \\
\text{IMP} & \rightarrow \text{AMP}
\end{align*}
\]

For simplicity, we do not include NH$_4$ in the AMPD reaction.

A.2. Figures

Data sources for figures 5 and 7 are A [27], B [28], C [29], D [30], E [31], F [32]. For repeated cases, the first case shows changes during ATP depletion and the second case shows changes during ATP regeneration.

In figure 6, (b) shows sensitivity with $K_a = 1$ and (c) shows sensitivity with $K_a = 1$ and $B_a = 1$.

Simulations of the model (2.1) in figure 1 use constant $p$, $r = g_2$, where $g = 0.1$, and $g = -b_1 y + b_{-1} x$ where $b_1 = 500$, $b_{-1} = 100$ for the buffering case (resulting in $B = 5$).

Simulations of the model (3.1) in figure 3 use $p = u_x$, $r = g y$ and $g = 0$, $r - p$, where $u_x$ is a control input (negative unit step) and $g$ is a kinetic constant.
