# Protons and Anaerobiosis

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*Summary.* During oxygen limitation in animals, glucose can be fermented via several metabolic pathways varying in energetic efficiency and leading to various end products (such as lactate, alanopine, octopine, succinate, or propionate). Because of opposite *p*H dependencies of proton production by fermentation and by hydrolysis of adenosine triphosphate formed in the fermentation, the total number of moles of protons generated is always two per mole of the fermentable substrate. However, two and three times more adenosine triphosphate can be turned over per mole of protons produced in succinate and propionate fermentations, respectively, than in lactate fermentation.

In normal aerobic metabolism, animals turn over impressive quantities of hydrogen ions. A simple calculation shows that during aerobic metabolism about eight hydrogen ions are formed per molecule of oxygen consumed, which means that in a 70-kilogram man, with a resting metabolic rate of about 700 millimoles of oxygen per hour, about 150 grams of $H^+$ are produced per day (*1*). The main sink for this vast amount of $H^+$ is oxidative phosphorylation, for there is normally a close balance between the rate of $H^+$ production and the rate of $H^+$ removal during adenosine triphosphate (ATP) resynthesis and reoxidation of reduced coenzymes and reduced cytochromes (*1*). Because of this balance, *p*H remains virtually constant during normal aerobic metabolism in both intracellular and extracellular fluids.

This closely balanced system dissipates during anaerobic metabolism, but how and why the breakdown occurs is controversial (*2, 3*). In part, the controversy arises from a common belief that glycolysis yields lactic acid, a fairly strong acid ($pK_a$, 3.9), which then dissociates into lactate anions and $H^+$. This oversimplified statement, which we found in four of five recent and successful biochemistry textbooks, is in fact rather misleading. Glycolysis per se represents the cleavage of glucose (or the glycogen-derived glucosyl unit) into two lactate anions with the concomitant production of ATP from adenosine diphosphate (ADP) and inorganic phosphate ($P_i$). If the overall reaction is, for a moment, considered in isolation and at high enough *p*H (> 8.0), it will be evident that $H^+$ neither need be accumulated nor depleted.

$$\text{Glucose} + 2\ ADP^{3-} + 2\ HPO_4^{2-} \longrightarrow 2\ \text{lactate}^- + 2\ H_2O + 2\ ATP^{4-} \quad (1)$$

The equation as written is balanced with regard to all constituents and charge. Only at low *p*H (< 6.0), well below physiological range, would glycolysis lead to the classical proton stoichiometry.

$$\text{Glucose} + 2\ HADP^{2-} + 2\ H_2PO_4^- \longrightarrow 2\ \text{lactate}^- + 2\ HATP^{3-} + 2\ H_2O + 2\ H^+ \quad (2)$$

Although the above equations illustrate important points, these simplest of situations are indeed never observed in living systems, largely because of three complicating factors: magnesium ions ($Mg^{2+}$), *p*H, and the substrate source (glucose or glycogen) being fermented (*4–6*) (Tables 1 to 7). Since *p*H, free $Mg^{2+}$ levels, and the availability of glucose rather than glycogen vary in different tissues, it is not possible to write a single equation for glycolysis in all tissues, nor even for the same tissue under differing conditions. This can be easily illustrated by considering anoxic vertebrate muscle. From the best evidence available, it appears that the concentration of free $Mg^{2+}$ in muscle is 3 to 4.4 m*M* (*7*), and this does not change substantially with activity. Over a reasonably broad range of *p*H (*5*), essentially all ATP is in the complexed form in vivo (*4*), ADP is only partially complexed (*4, 5, 7*), and $P_i$ binds $Mg^{2+}$ so weakly that it can be assumed under physiological conditions to be mostly uncomplexed (*5*). This means that the true adenylate reactants in glycolysis are strongly determined by $Mg^{2+}$ concentrations (Table 1, a and b), and since free $Mg^{2+}$ levels vary among different species and among tissues [0.25 m*M* is found in red blood cells, for example (*8*)], the overall equations for glycolysis may be quite cell-specific. Because the *pK* values of MgATP and free ATP are different, the amount of $H^+$ formed per mole of glucose fermented differs and, at any given *p*H, is greater if the ATP is fully complexed than if ATP is assumed to be fully uncomplexed (Table 1, a and b).

Whereas a potential role for $Mg^{2+}$ in the control of glycolytic $H^+$ production may be implied by these data, the implication is weakened by the observation that free $Mg^{2+}$ concentration in any given tissue does not appear to vary substantially with change in metabolic state [for example, levels do not change much during transitions from rest to work in muscle (*9*)]. However, this is not the case for *p*H, which is known to undergo reasonably large (and presumably controlled) changes during various metabolic transitions (*2–22*). The effect of *p*H change on glycolytic $H^+$ production can be illustrated by extending the example of anoxic vertebrate muscle—that is, fermenting glucose at varying *p*H but unchanging (4.4 m*M*) free $Mg^{2+}$ concentrations. Because the *pK* values for some of the reactants are close to the *p*H range at which the overall fermentation is occurring, it is necessary to write the equations for glycolysis with fractional charge numbers, or (our preference) with fractional stoichiometric coefficients. When this is done, a patent relationship is clarified between *p*H and the amount of $H^+$ formed per mole of glucose fermented to lactate: the lower the *p*H, the greater the amount of $H^+$ formed (Table 1, a and b).

Although the overall relationship is similar when glycogen is the starting fermentable substrate, the detailed situation differs substantially (because one $H^+$-producing step, that catalyzed by hexokinase, is not utilized). In this case (Table 2), at *p*H values only slightly above neutrality, glycogen fermentation to lactate proceeds with the consumption (not the production) of $H^+$. At the intracellular *p*H of 7.4 in frog muscle at 4°C (*4*), for example, 0.4 mole of $H^+$ is consumed per mole of glucosyl unit fermented. Since the proton stoichiometry seems adjustable, it is interesting to examine this problem in "good" animal anaerobes.

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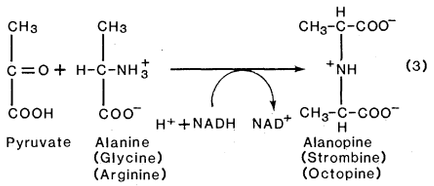
## Bivalve Glycolysis

Among animals highly tolerant to anoxia, certain mollusc groups are particularly well understood. In these organisms, the terminal step in anaerobic glycolysis is often modified, lactate dehydrogenase being omitted or occurring in low activity; in its stead, these organisms utilize octopine, alanopine, or strombine dehydrogenases (*11*)—or sometimes, all three (*12*). The reactions catalyzed are shown in Eq. 3. During




(3)

NAD+, nicotinamide adenine dinucleotide
NADH, reduced form

anaerobiosis in molluscs, therefore, lactate does not accumulate as a glycolytic end product; rather alanopine, strombine, or octopine do (*11, 13*). A close examination of the reaction pathways leading to these end products indicates that, in terms of proton production, they are essentially equivalent to mammalian glycolysis (Table 3). If alanopine or strombine were complexed with a divalent cation, possibly $Ca^{2+}$ derived from the shell [see (*14*)], the amount of $H^+$ produced per mole of glucose would be decreased, but whether or not this occurs in nature is not yet known. Where bivalve anaerobiosis greatly differs from that in vertebrates is in its capacity for, and utilization of, alternative fermentation pathways. Of these, the pathways of glucose fermentation either to succinate or to propionate are of particular importance.

## Succinate and Propionate as Anaerobic End Products

It is now well recognized that, during anoxia, many invertebrates (*14–16*) routinely accumulate succinate as a major anaerobic end product. Although the pathways to succinate may vary somewhat in different tissues and in different species, a common route involves the fermentation of glucose along the glycolytic path to the level of phosphoenolpyruvate (PEP); the latter is then carboxylated in a reaction catalyzed by the enzyme PEP carboxykinase to form oxaloacetate, which is subsequently reduced to succinate. The succinate-forming reaction is catalyzed by fumarate reductase and is thought to be ATP-yielding (*17, 18*); the overall energy yield of the glucose → succinate pathway is considered to be 4 moles of ATP per mole of glucose.

In some organisms, particularly in many parasitic helminths, succinate can be further metabolized to propionate with the further production of 1 mole of ATP per mole of propionate formed. The potential energy yield of this process is therefore 6 moles of ATP per mole of glucose—substantially higher than in vertebrate glycolysis (*15, 19*). But how does the $H^+$ stoichiometry compare? If we assume a free $Mg^{2+}$ concentration similar to that above, the overall equations for glucose fermentation to succinate and to propionate show that these pathways differ strikingly from classical glycolysis (Tables 4 and 5). For propionate production, for example, through probable physiological *p*H ranges for invertebrate ectotherms, the fermentation reaction proceeds with the consumption of $H^+$. If the starting substrate is glucose, at near neutral *p*H, about 1 mole of $H^+$ is consumed per mole of glucose (Table 5); at higher *p*H values, which may be expected in ectotherms at low temperatures (*23*), the molar yield of $H^+$ is even higher. Glycogen fermentation to propionate also consumes $H^+$, but in substantially larger amounts than when glucose is the starting substrate (Table 5). For this fermentation pathway, the *p*H must fall below ~ 6.5 before—on a molar basis (moles of $H^+$ per mole of glucose or glucosyl unit)—there occurs a net production of $H^+$.

## Functional Significance of Proton Stoichiometry

Since gradual acidification is a well-known consequence of sustained anoxia, the above data indicate that it would be advantageous (i) to utilize endogenous glycogen rather than glucose as the main fermentable substrate, and (ii) to utilize energetically efficient glycogen → succinate or glycogen → propionate fermentation reactions for meeting energy demands during $O_2$ limiting episodes. The first strategy is commonly observed in anoxia-tolerant tissues of higher animals, and the second is a hallmark strategy of many facultatively anaerobic invertebrates. In both cases, two advantages accrue. First, the energy yield (moles of ATP per mole of glucosyl unit) is greater than with simple glucose → lactate fermentation; and second, hydrogen ions are either actually consumed during the fermentation reaction or are generated at relatively low yields. These advantages are clear; yet there remains a problem, for it is evident that neither glycogen nor glucose fermentations in themselves can account for the well-known association between end-product accumulation and acidosis. Some of these fermentations actually consume protons. If the observed hydrogen ions are not formed primarily in the fermentation reactions, what is their source?

Table 1. Proton stoichiometry for anaerobic glycolysis at different values of *p*H, with or without $Mg^{2+}$. For all calculations in this and subsequent tables, the dissociation constants at 25°C for all relevant species are taken from (*5*). The enthalpy values for these constants, given in Alberty (*5*), are low; hence, the equations in the tables should be generally applicable for animals with widely differing body temperatures.

*a. Glucose → lactate, with no free $Mg^{2+}$*

*p*H 6.8

$$\text{Glucose} + 1.10\ ADP^{2-} + 0.90\ ADP^{3-} + 1.00\ P_i^- + 1.00\ P_i^{2-} \rightarrow 2\ \text{lactate}^- + 1.17\ ATP^{3-} + 0.83\ ATP^{4-} + 0.93\ H^+$$

*p*H 7.4

$$\text{Glucose} + 0.46\ ADP^{2-} + 1.54\ ADP^{3-} + 0.39\ P_i^- + 1.61\ P_i^{2-} \rightarrow 2\ \text{lactate}^- + 0.52\ ATP^{3-} + 1.48\ ATP^{4-} + 0.33\ H^+$$

*p*H 8.0

$$\text{Glucose} + 0.14\ ADP^{2-} + 1.86\ ADP^{3-} + 0.11\ P_i^- + 1.89\ P_i^{2-} \rightarrow 2\ \text{lactate}^- + 0.16\ ATP^{3-} + 1.84\ ATP^{4-} + 0.09\ H^+$$

*b. Glucose → lactate, with 4.4 mM free $Mg^{2+}$*

*p*H 6.8

$$\text{Glucose} + [1.36\ MgADP^- + 0.30\ ADP^{3-} + 0.30\ ADP^{2-} + 0.04\ MgADP + 0.86\ P_i^{2-} + 0.86\ P_i^- + 0.28\ MgP_i + 0.25\ Mg^{2+}] \rightarrow 2\ \text{lactate}^- + [1.91\ MgATP^{2-} + 0.04\ ATP^{4-} + 0.03\ ATP^{3-} + 0.02\ MgATP^-] + 1.15\ H^+$$

*p*H 7.40

$$\text{Glucose} + [1.56\ MgADP^- + 0.34\ ADP^{3-} + 0.09\ ADP^{2-} + 0.01\ MgADP + 1.26\ P_i^{2-} + 0.32\ P_i^- + 0.42\ MgP_i] \rightarrow 2\ \text{lactate}^- [1.94\ MgATP^{2-} + 0.04\ ATP^{4-} + 0.01\ ATP^{3-} + 0.01\ MgATP^- + 0.04\ Mg^{2+}] + 0.40\ H^+$$

*p*H 8.00

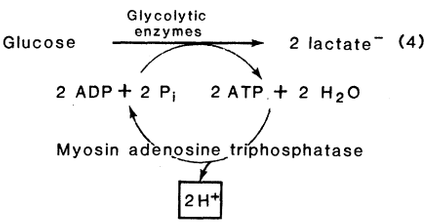
$$\text{Glucose} + [1.62\ MgADP^- + 0.36\ ADP^{3-} + 0.02\ ADP^{2-} + 1.43\ P_i^{2-} + 0.09\ P_i^- + 0.48\ MgP_i] \rightarrow 2\ \text{lactate}^- + [1.96\ MgATP^{2-} + 0.04\ ATP^{4-} + 0.14\ Mg^{2+}] + 0.11\ H^+$$

### ATP Hydrolysis and $H^+$ Production

During anoxic episodes, the fate of glycolytically generated ATP is hydrolysis for the support of various cellular work functions. As in aerobic metabolism, during anaerobiosis, the hydrolysis of ATP [by adenosine triphosphatase (ATPase) of myosin, for example, during muscle work] proceeds with the release of ADP, $P_i$, and $H^+$. The two main intracellular factors determining the amount of $H^+$ released during ATP hydrolysis again are $Mg^{2+}$ and $p$H (*5*). As before, because we are dealing with reactants whose $pK$ values are close to physiological levels of $p$H, fractional stoichiometric coefficients best describe the overall equations for ATP hydrolysis. For mammalian muscle (at constant 4.4 m$M$ free $Mg^{2+}$ levels), the equations (Table 6) show an interesting dependence on $p$H: over the range $p$H 6.5 to 8.0, the lower the $p$H, the fewer the moles of $H^+$ formed per mole of ATP hydrolyzed. This effect of $p$H on $H^+$ production during ATP hydrolysis, since it is almost exactly opposite to that observed for the glycolytic production of $H^+$, is fundamental because in vivo the two processes (glycolytic generation of ATP and ATPase-catalyzed hydrolysis of ATP) are, to some extent, coupled; neither proceeds in a vacuum. The adenylates are, of course, the basis for the coupling since ATP is one of the products of glycolysis at the same time as it is the key substrate for cell ATPases. If we momentarily assume very tight (or complete) coupling, it is easy to demonstrate the stoichiometry of net proton production by the two processes in total anoxia.

### Stoichiometry of Net Proton Production During Anaerobiosis

The coupling of ATP-forming and ATP-utilizing reactions is also a centerpiece of aerobic metabolism (*24*). However, this coupling in anaerobic glycolysis is different from that observed in aerobic metabolism. In the latter, all the products of ATP hydrolysis (ADP, $P_i$, and $H^+$) are reutilized during oxidative phosphorylation. In the former, by contrast, although ADP and $P_i$ are stoichiometrically reutilized during glycolytic replenishment of ATP, hydrogen ions are not reutilized if glucose is the fermentable substrate and are only partially reutilized if glycogen is being fermented above $p$H 7.2 (see Tables 1b and 2). Because of the opposite $p$H dependencies of $H^+$ production by glycolysis and by ATP hydrolysis, however, the total number of moles of $H^+$ generated (moles of $H^+$ per mole of glucose or glucosyl unit fermented plus moles of $H^+$ released during ATPase-catalyzed hydrolysis of the ATP formed during the fermentation) is always the same: two (Table 7). That is why for the overall system (glycolysis plus ATPase), the process can be described by one equation (Eq. 4)




with the stoichiometry (of proton production) being independent of $p$H and $Mg^{2+}$ levels. With glycogen as the starting substrate, although some $H^+$ may be consumed during fermentation, 3 rather than 2 moles of ATP per mole of glucosyl unit are generated, and when this greater amount of ATP is hydrolyzed by cell ATPases the net yield is 2 $H^+$ per glucosyl unit (Table 7).

An unexpected outcome of analyzing other animal fermentation pathways is that exactly the same stoichiometry of net $H^+$ production prevails. That is, the fermentation of either glucose or glycogen (i) to alanopine, strombine, or octopine, or (ii) to succinate or propionate, proceeds with a variable utilization or production of protons associated with ATP replenishment (Tables 1 to 5), but when this process is coupled to the hydrolysis of the ATP formed in the fermentation (Table 6), one equation (Eq. 5)

Table 2. Proton stoichiometry for glycogen fermentation to lactate, assuming 4.4 m$M$ free $Mg^{2+}$. Symbols: $a$, sum of all species of ADP and $P_i$ at indicated $p$H to unity; $b$, sum of all species of ATP at indicated $p$H to unity. Specific values for $a$ and $b$ are given in square brackets in Table 1b.

| |
|---|
| $p$H 6.80 |
| Glycogen + 3[$a_{6.8}$] → 2 lactate$^-$ + 3 [$b_{6.8}$] + 0.72 $H^+$ |
| $p$H 7.40 |
| Glycogen + 3 [$a_{7.4}$] + 0.40 $H^+$ → 2 lactate$^-$ + 3 [$b_{7.4}$] |
| $p$H 8.00 |
| Glycogen + 3 [$a_{8.0}$] + 0.84 $H^+$ → 2 lactate$^-$ + 3 [$b_{8.0}$] |

Table 3. Proton stoichiometry for alanopine formation, assuming 4.4 m$M$ free $Mg^{2+}$. For definition of $a$ and $b$, see Table 2.

| |
|---|
| $p$H 6.80 |
| Glucose + 2 alanine + 2 [$a_{6.8}$] → 2 alanopine$^-$ + 2 [$b_{6.8}$] + 1.15 $H^+$ |
| $p$H 7.40 |
| Glucose + 2 alanine + 2 [$a_{7.4}$] → 2 alanopine$^-$ + 2 [$b_{7.4}$] + 0.40 $H^+$ |
| Glycogen + 2 alanine + 3 [$a_{7.4}$] + 0.40 $H^+$ → 2 alanopine$^-$ + 3 [$b_{7.4}$] |
| $p$H 8.00 |
| Glucose + 2 alanine + 2 [$a_{8.0}$] → 2 alanopine$^-$ + 2 [$b_{8.0}$] + 0.11 $H^+$ |

Table 4. Proton stoichiometry for succinate formation, assuming 4.4 m$M$ free $Mg^{2+}$. For definitions of $a$ and $b$, see Table 2.

| |
|---|
| $p$H 6.80 |
| Glucose + 1.53 $HCO_3^-$ + 0.47 $H_2CO_3$ + 4 [$a_{6.8}$] → 2 succinate$^{2-}$ + 4 [$b_{6.8}$] + 0.77 $H^+$ |
| $p$H 7.4 |
| Glucose + 1.87 $HCO_3^-$ + 0.13 $H_2CO_3$ + 4 [$a_{7.4}$] + 1.07 $H^+$ → 2 succinate$^{2-}$ + 4 [$b_{7.4}$] |
| Glycogen + 1.87 $HCO_3^-$ + 0.13 $H_2CO_3$ + 5 [$a_{7.4}$] + 1.87 $H^+$ → 2 succinate$^{2-}$ + 5 [$b_{7.4}$] |
| $p$H 8.0 |
| Glucose + 1.97 $HCO_3^-$ + 0.03 $H_2CO_3$ + 4 [$a_{8.0}$] + 1.75 $H^+$ → 2 succinate$^{2-}$ + 4 [$b_{8.0}$] |

Table 5. Proton stoichiometry of propionate fermentation, assuming 4.4 m$M$ free $Mg^{2+}$. For definitions of $a$ and $b$, see Table 2.

| |
|---|
| $p$H 6.80 |
| Glucose + 6 [$a_{6.8}$] + 0.55 $H^+$ → 2 propionate$^-$ + 6 [$b_{6.8}$] |
| $p$H 7.40 |
| Glucose + 6 [$a_{7.4}$] + 2.80 $H^+$ → 2 propionate$^-$ + 6 [$b_{7.4}$] |
| Glycogen + 7 [$a_{7.4}$] + 3.60 $H^+$ → 2 propionate$^-$ + 7 [$b_{7.4}$] |
| $p$H 8.00 |
| Glucose + 6 [$a_{8.0}$] + 3.67 $H^+$ → 2 propionate$^-$ + 6 [$b_{8.0}$] |

always satisfactorily describes the net stoichiometry of proton production, and in all cases, net proton production is independent of *p*H. (In Eq. 5, *n* refers to the number of moles of ADP, $P_i$, or ATP per mole of glucose and varies with metabolic pathway.) That is why, in most animals, sustained anoxia results in a net accumulation of $H^+$ ions and why end-product accumulation and acidosis have often been correlated in the past. The metabolically more relevant relationship is between ATP and $H^+$; for the glucose → lactate fermentation, for example, 1 micromole of ATP is cycled through the system per micromole of $H^+$ accumulated, if 1:1 coupling with cell ATPases is assumed. For the fermentation of glycogen to lactate, 1.5 μmole of ATP are cycled through the system per micromole of $H^+$ accumulated. For the fermentation of glucose to succinate, 2 μmole of ATP are cycled per micromole of $H^+$ accumulated, and for the fermentation of glucose to propionate, that value increases to 3. This insight identifies the principal advantage gained by the utilization of succinate or propionate fermentations by good invertebrate anaerobes; during anoxia, up to three times more ATP can be turned over per mole of $H^+$ accumulated than is the case for mammalian tissues relying solely on glycolysis.

### Degree of Coupling Between Glycolysis and ATP Hydrolysis

Important consequences stem from the way in which the anaerobic ATP-generating pathways are coupled to cell ATPase functions. In the extreme case above of tight (1:1) coupling, the proton stoichiometry during sustained anoxia is independent of the kind of fermentation pathways utilized: 2 moles of $H^+$ per mole of glucosyl unit are formed by them all. Since the fermentative and the ATPase functions are spatially separate (catalyzed by different enzyme pathways), temporally independent operation would represent the other extreme situation of uncoupled function. In anoxic muscle, for example, this situation might lead to a depletion of ATP (by myosin ATPase catalysis during anaerobic work) before activation of glycolysis. In nature, under usual circumstances, neither extreme is observed; activation of fermentative reactions and of ATPase are fairly closely coordinated, so that increased work (increased ATP turnover) usually occurs with only modest changes in ATP concentrations, in both vertebrate and invertebrate systems. Small oscillations, implying some temporal separation, are, in fact, considered thermodynamically advantageous (*25*). However, during drastic rise in ATP requirements—for example, in humans at their limits of performance (*26*), in invertebrates during sustained anoxia coupled with work demands (*27*), and so forth—the rates of ATP utilization surpass rates of production and ATP levels fall substantially.

Table 6. Stoichiometry of ATP hydrolysis under different *p*H conditions. For definitions of *a* and *b*, see Table 2.

*p*H 6.80
$[b_{6.8}] + H_2O \rightarrow [a_{6.8}] + 0.425\ H^+$

*p*H 7.40
$[b_{7.4}] + H_2O \rightarrow [a_{7.4}] + 0.80\ H^+$

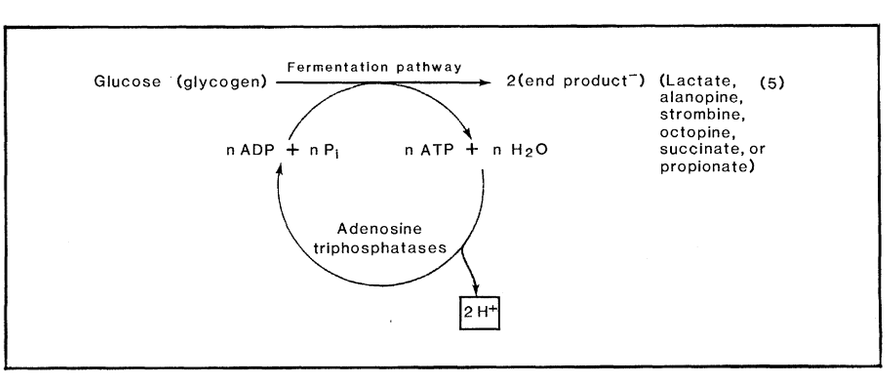
*p*H 8.00
$[b_{8.0}] + H_2O \rightarrow [a_{8.0}] + 0.945\ H^+$

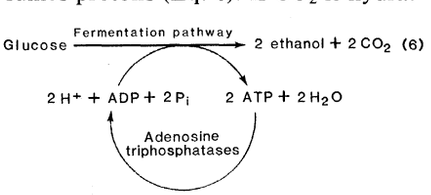
In humans, at extreme work levels, muscle ATP concentrations fall from initial levels of about 5 μmole to about 2.5 μmole per gram (*26*); in salmonids forced to swim at maximum rates for maximum (about 45 minutes) time periods, ATP concentrations in white muscle fall by a surprisingly large amount, from about 7 to 2 μmole per gram (*26*). All such cases represent a certain amount of uncoupling between myosin ATPase function and fermentative ATP replenishment, so that proton production rates must become unusually high. In humans, the drop in ATP levels represents a potential increase in $H^+$ concentrations from about 0.1 nmole to 2.5 μmole per gram; in the absence of protective buffering mechanisms, this would represent an increase of several orders of magnitude in $H^+$ levels. Under these conditions, there may be a selective advantage to retaining $H^+$-consuming processes, such as adenosine monophosphate (AMP) deamination to inosine monophosphate (IMP) plus $NH_3$ (which is protonated to $NH_4^+$), a reaction catalyzed by AMP deaminases and characteristic of vertebrate muscles. However, this reaction sequence depletes the adenylate pool while contributing nothing to the energy status of the anoxic cell.

Thus where the urgent need is to absorb or consume protons, the best solution is to utilize fermentative pathways that consume $H^+$ during ATP replenishment: for example, glycogen → succinate or glycogen → propionate. The advantage of these kinds of reaction paths [over the simple fermentation of glucose to lactate, which produces rather than consumes protons (Table 1)] is that they at least partially redress the overproduction of $H^+$ by ATP hydrolysis. This advantage seems so obvious that a biologist might wonder why fermentation pathways leading to no net change in $H^+$ balance in the coupled system have not been developed. It turns out that they have: in the fermentation of glucose to ethanol [used by many microorganisms and by some animals (*28*)] and in the fermentation of glucose to butanol [used by some microorganisms (*29*)].

### Alcohol Fermentations

The distinct advantage of alcohol fermentation is that it can proceed with $H^+$ consumption equivalent to $H^+$ production by ATP hydrolysis; when coupled with cell ATPases, the system as in aerobiosis neither produces nor consumes protons (Eq. 6). If $CO_2$ is hydrated, its production may become equivalent to the production of 2 $H^+$ per mole of glucose used up (Table 8), in effect negating the advantage of the system. That is why organisms utilizing this scheme must also provide a means for removing $CO_2$. In the anoxic goldfish (*28*), the probable solution is removal of $CO_2$ across the gill into the external water, which serves as a large $CO_2$ sink



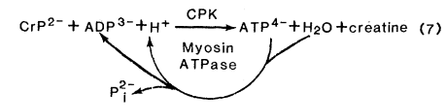



for aquatic animals. Similar conditions for $CO_2$ release apply for *Chironomus* larvae (*30*) and various endoparasites (*31*) that produce ethanol during $O_2$ limitation. In the case of butanol formation in microorganisms, the pathway becomes activated only at acidic *p*H when $CO_2$ hydration would not be favored and $CO_2$ along with another gas, $H_2$, again can be removed to the external medium (*29*). The advantages of these pathways do not come without cost; namely, loss to the medium of a carbon source (the end product), which could be utilized for oxidative energy metabolism during aerobic recovery (*28*). Nevertheless, it is clear that nature can and has invented fermentation pathways that consume the protons released during ATP hydrolysis. The paradox is that such pathways are actually rather rarely used by good animal anaerobes; only goldfish and a few invertebrate species (*28, 30, 31*) currently are known to employ this strategy of anoxia adaptation. One possible reason why it is not more generally used is that its advantages are compromised because ethanol and other short-chain alcohols diffuse freely between body compartments and thus their distribution cannot be readily regulated (*28*). Another possibility, which we feel needs to be explored, is that in standard animal systems there may be important but thus far largely unrecognized functions for net proton production during $O_2$ limiting periods.

From currently available data, we can suggest four such roles for $H^+$ accumulation during $O_2$ limitation: (i) creating conditions more favorable to unloading whatever $O_2$ remains in the organism (that is, the Bohr shift), (ii) facilitating phosphagen hydrolysis, (iii) establishing a *p*H optimum for glycolysis or at least regulating the glycolytic rate, and (iv) facilitating lactate (or end-product) efflux. The physiological and metabolic implications of the Bohr shift are well understood and are available in any standard textbook. However, (ii), (iii), and (iv) need to be more closely examined, since the most abundant data on muscle (to which we shall restrict ourselves for the remainder of this article) are controversial and have not been considered in this framework.

## Phosphagen Support of Muscle Work

The first phase of muscle work during anoxia is supported by creatine phosphate (CrP) hydrolysis in vertebrates and arginine phosphate (ArgP) hydrolysis in many invertebrates. The role of the phosphagen is classically considered to be that of an ATP buffer, ATP being formed by transphosphorylation from the phosphagen as fast as it is being split by myosin ATPase. At a maximum power output of about 3 μmole of ATP per gram per minute (*32*), all CrP reserves (about 30 μmole per gram) can be depleted in about 10 seconds of muscle work. At least during early stages of this process, creatine phosphokinase (CPK) activation apparently depends on an increased availability of ADP (*33*) and presumably $H^+$, both derived from ATP hydrolysis. Since $H^+$ is in effect a substrate for the forward CPK reaction (but occurs at only about $10^{-7}M$ concentrations), initial production from ATP hydrolysis may serve as a means for integrating CPK and myosin ATPase functions so closely that minimal change in ATP concentration occurs at least during early stages of activation (*33*) (Eq. 7). Because of the influence of $Mg^{2+}$, the actual proton stoichiometry is somewhat more complex (Table 9). On balance,




however, the coupling of phosphagen hydrolysis with ATPase functions would lead to a slight alkalinization, thus (together with decreasing CrP concentration) supplying an automatic means for subsequent slowing down of the CPK reaction. Exactly the same situation applies for arginine phosphate or the hydrolysis of other phosphagens (Table 9).

In most molluscs studied up to now, there is an easily measurable separation in time between maximum rates of phos-

Table 7. Proton stoichiometry of various fermentation pathways coupled to cell ATPases. *A*, $H^+$ formed in fermentation expressed as moles of $H^+$ per mole of glucosyl unit. *B*, moles of $H^+$ obtained by hydrolysis of ATP formed in fermentation. *A* + *B*, moles of $H^+$ per mole glucosyl unit plus moles of $H^+$ from hydrolysis of ATP formed in fermentation.

| Fermentation process | *p*H | *A* | *B** | *A* + *B* |
|---|---|---|---|---|
| Glucose → lactate | 6.8 | 1.15 | 0.85 (2) | 2.00 |
| | 7.4 | 0.40 | 1.60 (2) | 2.00 |
| | 8.0 | 0.11 | 1.89 (2) | 2.00 |
| Glycogen → lactate | 6.8 | 0.72 | 1.28 (3) | 2.00 |
| | 7.4 | −0.40 | 2.40 (3) | 2.00 |
| | 8.0 | −0.84 | 2.84 (3) | 2.00 |
| Glucose → alanopine | 7.4 | 0.40 | 1.60 (2) | 2.00 |
| Glucose → propionate | 7.4 | −2.80 | 4.80 (6) | 2.00 |
| Glucose → succinate | 7.4 | −1.07 | 3.20 (4) | 2.13 |

*Numbers in parentheses refer to assumed moles ATP formed per mole glucosyl unit during the fermentation process.

Table 8. Proton stoichiometry of ethanol production with and without $CO_2$ hydration. For definition of *a* and *b*, see Table 2.

*p*H 7.4

Glucose + 2 $[a_{7.4}]$ → 2 ethanol + $[b_{7.4}]$ + 1.87 $HCO_3^-$ + 0.13 $H_2CO_3$ + 0.27 $H^+$

Glycogen + 3 $[a_{7.4}]$ + 0.53 $H^+$ → 2 ethanol + 3 $[b_{7.4}]$ + 1.87 $HCO_3^-$ + 0.13 $H_2CO_3$

Glucose + 2 $[a_{7.4}]$ + 1.60 $H^+$ → 2 ethanol + 2 $[b_{7.4}]$ + 2 $CO_2$ (removed)

Table 9. Stoichiometry of phosphagen hydrolysis, with 4.4 m*M* free $Mg^{2+}$.

*a. Phosphocreatine*

*p*H 6.8

0.99 $CrP^{2-}$ + 0.01 $CrP^-$ + 0.68 $MgADP^-$ + 0.15 $ADP^{3-}$ + 0.15 $ADP^{2-}$ + 0.02 MgADP + 0.26 $Mg^{2+}$ + 0.85 $H^+$ → creatine + 0.95 $MgATP^{2-}$ + 0.02 $ATP^{4-}$ + 0.02 $ATP^{3-}$ + 0.01 $MgATP^-$

*p*H 7.4

1.00 $CrP^{2-}$ + 0.78 $MgADP^-$ + 0.17 $ADP^{3-}$ + 0.04 $ADP^{2-}$ + 0.01 MgADP + 0.19 $Mg^{2+}$ + 0.95 $H^+$ → creatine + 0.98 $MgATP^{2-}$ + 0.02 $ATP^{4-}$

*p*H 8.0

1.00 $CrP^{2-}$ + 0.81 $MgADP^-$ + 0.18 $ADP^{3-}$ + 0.01 $ADP^{2-}$ + 0.17 $Mg^{2+}$ + 0.99 $H^+$ → creatine + 0.98 $MgATP^{2-}$ + 0.02 $ATP^{4-}$

*b. Phosphoarginine*

*p*H 7.4

1.00 $ArgP^-$ + 0.78 $MgADP^-$ + 0.17 $ADP^{3-}$ + 0.04 $ADP^{2-}$ + 0.01 MgADP + 0.19 $Mg^{2+}$ + 0.95 $H^+$ → arginine$^+$ + 0.98 $MgADP^{2-}$ + 0.02 $ATP^{4-}$

phagen hydrolysis and of glycolysis (*11, 13*); this is also evident in muscles of cold-blooded vertebrates (*34*), but it is harder to see in mammals (*35*), perhaps because of higher metabolic rates. The physiological significance of this separation is clear; phosphagen hydrolysis, yielding the highest power output of any metabolic system (*32*), is turned on before glycolysis, which may be required as a backup system of ATP generation when phosphagen reserves are depleted. But what maintains dampened glycolytic rates during this time? Or put another way, what activates glycolysis as phosphagen supplies are running down? Although there may be several important hormonal or metabolite signals (*32, 36–38*), one possibility invokes a role for $H^+$; with the depletion of phosphagen, continued ATP demands may lead to myosin ATPase rates outstripping ATP replenishing reactions, at which time *p*H must fall. In rat skeletal muscle, with a buffering capacity of 68 μmole per *p*H unit per gram (*37*) and a maximum power output based on glycolysis of 1 μmole of ATP per gram per second (*32*), intracellular *p*H would drop from a normothermic resting value of 6.9 (*37*) to 5.9 in 68 seconds (assuming 1 μmole of $H^+$ per micromole of ATP turned over). This large a decrease in *p*H probably never occurs; nevertheless, some acidification is the rule during anaerobic glycolysis in working muscle (*33, 35, 36*). A metabolic function for protons at this time may well be to bring the intracellular *p*H to an optimum range for anaerobic glycolysis, allowing its full catalytic potential to be realized.

### The Interaction Between *p*H and Glycolysis

Since, in both vertebrates and invertebrates, activation of ATP hydrolysis during anoxia may precede glycolytic activation, it would be advantageous for glycolytic activation mechanisms to be designed either to be augmented by $H^+$ during these stages of anoxia, or at least to be fairly insensitive to them; otherwise the rate of ATP replenishment would immediately begin falling as the need for ATP by working muscle was rising. Although there are some data implying that small drops in *p*H may stimulate anaerobic glycolysis [see (*19–22*)], most previous studies have concluded that decreasing *p*H is essentially inhibitory. No one doubts that it is inhibitory if the *p*H drop is large enough; this is indicated by (i) inverse relationships between force that can be generated by isolated muscle and $H^+$ concentration (*4*), (ii) a correlation between *p*H drop and fatigue (*36*), and (iii) reduced rates of glycolysis when *p*H drops too much (*37*). However, as shown by Trivedi and Danforth (*20*), both the positive modulator, adenosine monophosphate, and the substrate lower the *p*H optimum for phosphofructokinase, which is considered the most *p*H-sensitive locus in glycolysis (*37*). Moreover, reexamination of data on isolated frog sartorius muscle shows a large production of $H^+$ (indicated by ATP depletion, assuming the stoichiometry in Table 6) before significant glycolytic activation; furthermore, once activated, glycolysis is sustained at unchanging rates for the entire 15-minute anoxic work period tested (*36*), accompanied by continued $H^+$ production and decreasing *p*H. Similarly, working with isolated preparations of mammalian muscle (rat extensor digitorum longus), Sahlin *et al.* (*35*) observed that during electrical stimulation prolonged until fatigue, the sharpest rise in $H^+$ concentration occurs during the first minute (when a notable decrease in ATP concentration occurs), during which time the preparation sustains the highest glycolytic rates; over the subsequent 4-minute period until fatigue, glycolytic rates remain almost unchanging despite continuous $H^+$ production and accumulation. Similarly, in studies in vivo of human subjects, voluntary contractions at 68 percent of maximum force cause a modest drop in ATP concentration, a consequent rise in $H^+$ concentration, and an overall large activation of glycolysis (*38*); in contrast, a 15-minute circulatory occlusion of the same muscle maintained at rest causes no change in ATP concentration, almost no change in *p*H, and minimum lactate formation and accumulation (*38*). Interestingly, during endotoxin-induced shock in mammals, Bastiaans and Vleeming (*21*) observed increasing rates of muscle glycolysis with decreasing *p*H. We have argued (*19*) that such data are consistent with either proton activation of glycolysis, particularly early in anoxia, or at least no necessary inhibitory effects. However, the experimental protocols in these kinds of studies usually have not been designed to clarify the interactions between *p*H and glycolysis. In addition, the intracellular *p*H may have been reduced even before measurements began as a result of experimental manipulations. Thus, for a variety of reasons, proton-mediated augmentation of glycolysis preceding the usually reported inhibitory effects may have been overlooked. That such occurs in yeast cells has been convincingly demonstrated by Gillies *et al.* (*22*), and we believe this possibility is in need of very careful reconsideration and testing in animal systems as well.

### The Control of Lactate Efflux

Another possible physiological function of generating $H^+$ during anoxia may relate to the problem of effluxing lactate. For many decades now it has generally been assumed that lactate exchange between body compartments is achieved by simple diffusion down concentration gradients. Recently, however, it has become apparent that, in at least a few tissues, lactate transfer is carrier-mediated. In the proximal tubule of the kidney it appears to be linked to $Na^+$ transport (*39*). In all other tissues examined, including several tumor lines (*40, 41*), red blood cells (*42*), liver (*43*), and skeletal muscle and heart (*44*), the carrier-mediated transfer of lactate is *p*H-dependent and appears to be an antiport system, lactate anions exchanging for $OH^-$ ions. Thus, we are led to the hypothesis that another function of generating $H^+$ ions inside muscle cells during anaerobiosis is to create conditions more suitable for lactate efflux, both during hypoxic episodes and during recovery from them.

### Role of Intracellular Buffers

From these considerations, it appears that anoxia in animals may require a compromise between balancing benefits obtainable from initial $H^+$ release and the risks of acidification. In that event, adaptational processes might have arisen designed to prevent unbridled acidification during anoxia. There is excellent evidence for just such adaptational processes. For over two decades it has been known that imidazole-based intracellular buffers in vertebrate muscles (carnosine, anserine, or histidine, depending on the species) occur in higher concentration in highly glycolytic muscles (*45*). In more recent studies of this problem in muscle (*46*), a fairly good correlation was pointed out between the catalytic potential of $M_4$ lactate dehydrogenase isozyme and buffering capacity in a wide selection of vertebrates. Comparisons of several fishes varying in burst swimming capacities indicate a similar general trend; for example, concentrations of histidine plus anserine in tuna white muscle, displaying a potent glycolytic capacity, are about

100 times higher than in muscle of sluggish flatfish (*47*).

All of the above studies were prompted by the assumption that dissociation of lactic acid is the source of $H^+$ during anoxia; hence, all of them were done with the expectation of finding correlations between glycolytic capacity and buffering. Since $H^+$ is produced mainly during muscle contraction, the two functions most likely to coadapt are myosin ATPase catalytic potential and buffering capacity, the correlation with glycolysis being of a secondary nature. We have not been able to find any studies bearing specifically on this question. However, supporting indirect evidence is available. Thus, muscles displaying highest activities of myosin ATPase are of the fast-twitch glycolytic type, and these contain the highest concentrations of carnosine or anserine (*48*). Furthermore, species such as the cat (adapted for burst performance) display higher levels of carnosine plus anserine than do less active animals (*48*). At least qualitatively, then, the above relationships seem to be realized.

## Conclusions

We are proposing four simple hypotheses applicable to both vertebrate and invertebrate systems: (i) that essentially all animals in anoxia generate 2 $H^+$ per mole of glucosyl unit when the ATP formed in fermentation is hydrolyzed to ADP and $P_i$, a stoichiometry independent of *p*H; (ii) that $H^+$ produced during early stages of anoxia favors phosphagen hydrolysis and in later stages may serve to establish an optimum *p*H for backup fermentative reactions; (iii) that, in later stages of anoxia and in recovery, $H^+$ accumulation creates conditions suitable for end-product efflux from muscle into blood; and (iv) that unbridled acidification is prevented by maintaining closely controlled proportions between the catalytic potential of cellular ATPase and imidazole-based intracellular buffering capacity.

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