Metabolic Consequences of Diving in Animals and Man

The diving habit calls for controlled oscillation between aerobic and anaerobic metabolism.

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The dramatic differences in underwater capabilities between vertebrate divers and nondivers have long intrigued layman and biologist alike and have led to the establishment of a sound understanding of the physiological and morphological adjustments that support the diving habit (*1*). Although we are not as knowledgeable about the metabolic and enzymatic adaptations of diving animals, the overall organization of metabolism is fairly clear. Thus, it is well known that in the normal, nondiving state the preferred fuel is fat. Values of *RQ* (the ratio of respiratory CO_2 produced to O_2 consumed), where they have been measured (*2*), are typically around 0.7, and the free fatty acid content of the blood is high (*3*). Both observations indicate a potentially high fat catabolism. Many diving vertebrates in fact display functional and anatomical adaptations favoring an active aerobic metabolism. Per unit body weight, diving vertebrates display larger lung volumes (*4*) and they have a large blood volume, unusually large blood O_2 capacities, and higher muscle myoglobin concentrations (*1*). Diving animals such as seals and whales have unusually large blood storage capacities in their venous systems (*1*). The proportion of red, mitochondria-rich muscle fibers with high lipase activity is high (*5*). All such adaptations imply that the aquatic vertebrate sustains a vigorous fat-based metabolism when not diving. It is fat-based (i) because many divers use blubber as thermal insulation and thus may have been "preadapted" for fat utilization, (ii) because fat is the most convenient fuel for sustaining the long migrations that are typically made by many marine mammals and birds, and (iii) because on a weight or a molar basis fat is nature's most energetically efficient fuel source (*6*).

In contrast to the nondiving state, deep diving is supported by an impressive glycogen-based fermentation, particularly in tissues such as skeletal muscle. Muscle is cut off from the blood circulation during diving by peripheral vasoconstriction, and its anaerobic metabolic capacities undoubtedly set broad limits on the physical work performed during long dives. In the heart and brain the situation is more complex. During the first phases of deep diving two processes, slowing of the heart rate (bradycardia) and extensive peripheral vasoconstriction (*1*), conserve O_2 for these two normally aerobic tissues and greatly extend the breath-hold period that can be maintained. In the terminal stages of prolonged diving, however, even these organs must tolerate anoxia for surprisingly long times (*7, 8*), and they typically store unusually large amounts of glycogen for this purpose (*9*). Taken together, the O_2 conservation mechanisms plus the increased glycolytic capacity of the heart and brain appear to set broad limits on the duration of diving, and they are remarkably effective. Compared to a trained human skin diver, who can submerge for only 1 to 2 minutes, the common harbor seal can dive for about 20 minutes, the antarctic Weddell seal for about an hour, the bottle-nosed whale for about 2 hours, and the red-eared turtle for several days (*1*). These are impressive achievements, and they clearly depend on a glycogen-based fermentation in most tissues of the diving animal. Accordingly, as interest in the field gradually shifted from the physiological level to the underlying biochemical adaptations, the first studies tended to focus on anaerobic metabolism. That was as it should have been. How, after all, does nature build "anaerobic machines" as effective as those observed in the best of the diving vertebrates?

It is now axiomatic that in all cells able to sustain significant periods of anoxia some provision is made (i) for a storage form of energy, (ii) for the maintenance of redox balance (that is, preventing the cell from passing into a highly reduced state), and (iii) for the generation of high-energy phosphate compounds, or adenosine triphosphate (ATP) equivalents. In divers, these three metabolic requirements are met by anaerobic glycolysis (Fig. 1), where glycogen, the storage form of energy, is catabolized to the level of lactate during anaerobiosis. Redox balance is maintained by a functional 1 : 1 integration of glyceraldehyde-3-phosphate dehydrogenase (*10*) and lactate dehydrogenase, catalyzing oxidative and reductive steps, respectively. Useful energy production in the form of ATP occurs at the phosphoglycerate kinase and pyruvate kinase reaction steps, a net of 2 moles of ATP being formed per mole of glucose. Given this overall system, how do "good" vertebrate anaerobes maximize it, or at least improve it, to support work under stressful conditions such as diving? Nature fortunately gives us some readily available clues and a good model system in vertebrate white muscle, which during burst activity derives most of its useful energy from anaerobic mechanisms (*11*). Before these can be fully appreciated, it is important to briefly review our current understanding of overall organization and control of muscle glycolysis.

Multiple Control Sites in Muscle Glycogenolysis

Over the last decade or so, it has become apparent that control of glycolysis never resides at one key site—the so-called "master reaction" of earlier literature. Rather, the pathway is broken up into various segments, in each of which there appears to be a key control reaction; during operation, control

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is transferred from site to site along the pathway, but we are not yet certain how this is organized in time (*12*). Is it sequential? Is it alternating, hopping forward, then backward, then forward again? We do not know which alternative is correct. What we do know with a high degree of certainty, however, is that three key control sites appear over and over again in different species and in different tissues (*13*). The three sites are (i) glycogen phosphorylase, (ii) phosphofructokinase, and (iii) pyruvate kinase, and in situations where glucose is an important alternate substrate for the pathway, hexokinase appears as an important regulatory site. These enzymes share an important characteristic: under physiological conditions they catalyze reactions that are, in effect, irreversible, for either thermodynamic or kinetic reasons, or both. Moreover, they occur in much higher titer in muscle than in other tissues and thus in part account for its greater glycolytic capacity (*14*). But a higher amount of key enzymes does not account for the capacity of muscle glycolysis to swing from a low activity state almost instantaneously to a several hundredfold activated state (*15*). This characteristic, unique to skeletal muscle, depends not on the amount of any given glycolytic enzyme, but on the kind of enzyme present; that is, on its specific regulatory properties. Thus, most of the glycolytic enzymes in muscle occur not only in high titer, but also in more or less tissue-specific form; understanding the way in which the three key enzymes of glycogenolysis are controlled and integrated should go a long way toward explaining why muscle glycolysis is such a good anaerobic machine.

Strategic Positioning and Control Functions of Glycogen Phosphorylase

As is evident in Fig. 1, glycogen phosphorylase is metabolically strategically positioned at the first step in the pathway and it initiates glycogen mobilization. It also appears to be physically strategically placed onto glycogen granules (*16*). From much in vivo and in vitro experimentation, it is now known to be under hormonal control (via a cascade control system) as well as under calcium control. The time course of phosphorylase activation by hormones is of the order of minutes, while calcium activation, which depends on muscle membrane depolarization and can therefore be thought of as a neuron-dependent activation mechanism, occurs in seconds or less (*17*). The calcium activation (Fig. 2) appears to be of primary importance (particularly to diving vertebrates, whose blood flow, and hence hormonal signals to muscle, are cut off during the dive).

The conversion of phosphorylase b to phosphorylase a is now considered to be central to glycogen mobilization in all vertebrate tissues, including muscle (*17*), but once the enzyme is in the active form, its activity still depends on substrate and modulator concentrations. In muscle, the affinity of phosphorylase a for glycogen is unusually high, 50 times higher, for example, than in the case of the brain enzyme (*18*), and the muscle enzyme can therefore bind substrate with high efficiency. Moreover, the enzyme is further activated by adenosine monophosphate (fivefold activation with 0.1 millimolar AMP) which increases enzyme-substrate affinity still further. These kinetic properties favor glycogen phosphorylase function in the glycolytic direction despite the thermodynamically "uphill" nature of the reaction and can set the stage for a glycolytic activation in muscle which surpasses by far that in any other tissue in the vertebrate body. To achieve this, the regulation of phosphorylase must be at least partially integrated with that of the next key control site in the pathway, 6-phosphofructokinase.

Autocatalytic Activation of Muscle Phosphofructokinase

Phosphofructokinase catalyzes the first committed step in glycolysis and hence has long been recognized as perhaps the single most important control site in glycolysis (*19*). A sufficient number of vertebrate tissues and species have now been examined to reveal a common control principle underlying all vertebrate phosphofructokinases. Thus for this enzyme the two cosubstrates, fructose-6-phosphate and ATP, serve both as substrates per se and as metabolite modulators. One substrate, fructose-6-phosphate, behaves as a typical positive modulator, while the cosubstrate, ATP, behaves as an important negative modulator. All other regulatory metabolites exert their effects either by modifying enzyme–fructose-6-phosphate affinity, or by modifying enzyme sensitivity to ATP, or more usually by modifying both fructose-6-phosphate and ATP saturation kinetics.

After the activation of glycogen phosphorylase and myofibrillar adenosine triphosphatase in normal skeletal muscle, rising concentrations of fructose-6-phosphate, fructose-1,6-diphosphate, adenosine diphosphate (ADP), AMP, and inorganic phosphate coupled with falling concentrations of creatine phosphate and ATP can lead to a very large "flare-up" of phosphofructokinase when it is required (*20*). A central feature of this control system is that two of the positive modulators are products of the reaction; one of the positive modulators is, of course, a substrate. Taken together, their regulatory effects lead to an autocatalytic, exponential rate of change from low activity states to high activity states, a characteristic that often is not seen in other cells (*21, 22*) and that helps to explain the speed with which muscle glycolysis is "turned on." Aside from this characteristic, other distinguishing features of muscle phosphofructokinase compared to that in tissues such as the liver include an overall "tighter" control by most organophosphate modulators and by citrate but a highly reduced sensitivity to ATP inhibition. Thus, for a given percentage change, muscle phosphofructokinase requires about one-tenth as much ADP, AMP, or citrate as does the liver homolog; at the same time, it requires two to three times higher ATP concentrations to bring about the same percentage inhibition (*23*). As we shall argue below, some of the muscle phosphofructokinase characteristics are further accentuated in vertebrate divers.

Finally, we wish to know how the phosphofructokinase catalytic rate is integrated with the activity of pyruvate kinase, the next major regulatory enzyme in glycolysis. In most mammals studied, the major integration mechanism merely involves adenylate coupling; that is, ADP, the product of the phosphofructokinase reaction, is a substrate for pyruvate kinase, and this in itself serves to automatically coordinate the activities of these two enzymes. In lower vertebrates (fishes and reptiles), pyruvate kinase is an allosteric enzyme under close metabolite regulation, and fructose-6-phosphate, the other product of the phosphofructokinase reaction, serves as a potent feedforward activator of muscle pyruvate kinase, assuring nearly simultaneous activation of both enzymes (*24*).

Maintenance of Redox Balance during Anaerobic Glycolysis in Muscle

In general, the redox balance (NAD/NADH, the ratio of oxidized to reduced nicotinamide adenine dinucleotide) during anaerobic glycolysis in muscle is maintained by a functionally 1 : 1 activity ratio between glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase, and this surely is the situation during early phases of glycolysis. Both enzymes occur in higher titer in vertebrate muscle than in other tissues (*14*), and in rat muscle, for example, they occur in about a 1 : 1 ratio. In extreme situations, the lactate dehydrogenase reaction becomes limiting by mass action effects (*25*); at this time, if muscle glycolysis is to continue, it must have some other source of NAD. That source is usually considered to be the α-glycerophosphate dehydrogenase reaction, in which case, α-glycerophosphate accumulates as an additional anaerobic end product (*12, 15, 26*). Presumably, some controlling mechanism is available to turn on α-glycerophosphate dehydrogenase at the correct time, but as of this writing we are aware of nothing definitive on this matter.

Not only is the amount of lactate dehydrogenase present in skeletal muscle adjusted for a high glycolytic potential, so is the kind of lactate dehydrogenase present. Thus, it is now widely accepted (*27*) that the muscle type of lactate dehydrogenase tetramer shows at least two kinetic properties which suit it admirably for function in a tissue of high glycolytic potential: low enzyme-substrate affinity and low substrate inhibition. The latter effect prevents substrate inhibition of the reaction during rapid glycolysis, while the low enzyme-substrate affinity prevents pyruvate saturation of the enzyme and hence prevents pyruvate accumulation

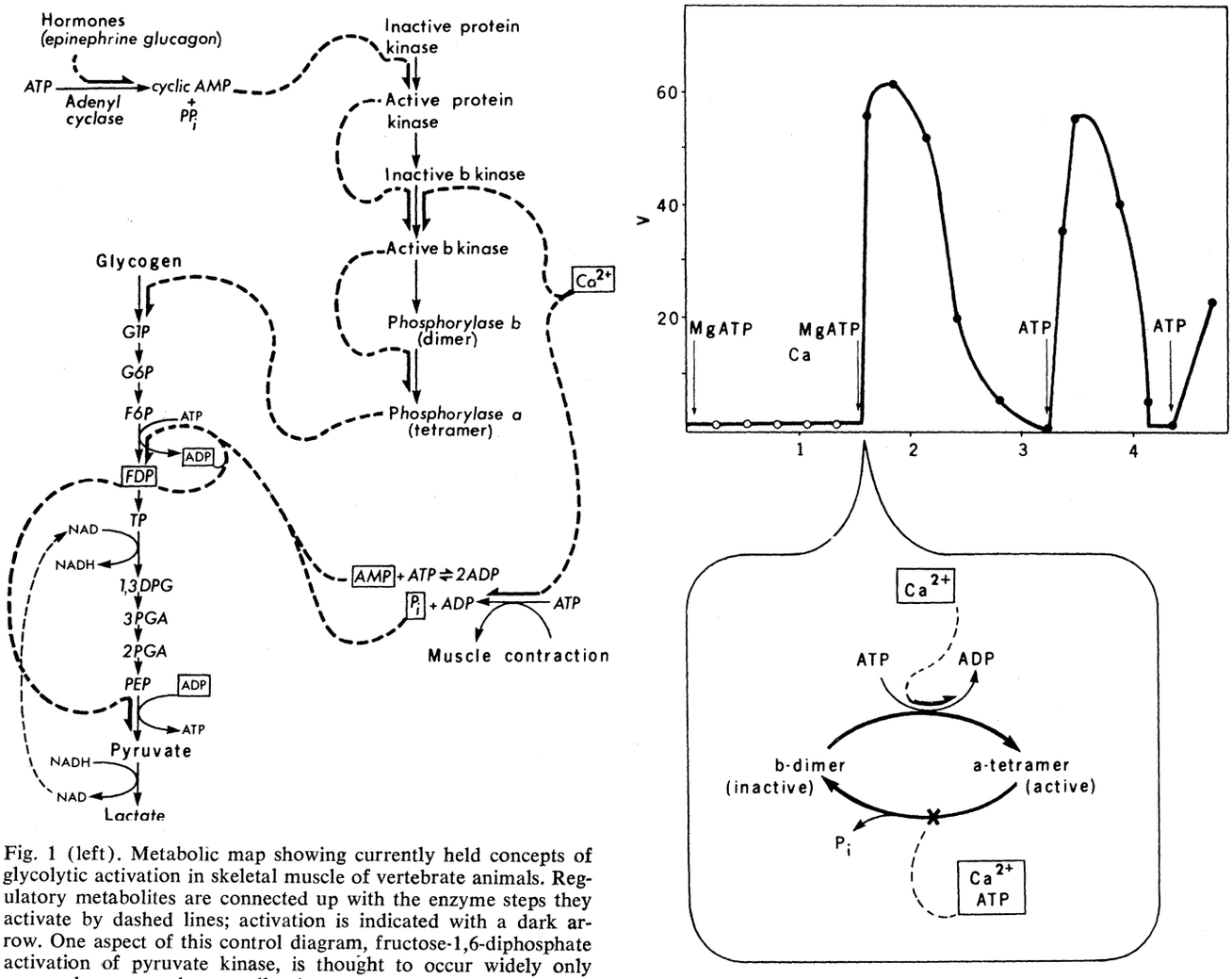

Fig. 1 (left). Metabolic map showing currently held concepts of glycolytic activation in skeletal muscle of vertebrate animals. Regulatory metabolites are connected up with the enzyme steps they activate by dashed lines; activation is indicated with a dark arrow. One aspect of this control diagram, fructose-1,6-diphosphate activation of pyruvate kinase, is thought to occur widely only among lower vertebrates; all other aspects are thought to be general, although certain characteristics are adjusted in diving vertebrates. [From Hochachka and Somero (*6*)] Abbreviations, used in Figs. 1 to 4, are: G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; TP, triose phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenol pyruvate; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate, respectively; NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide; P_i and PP_i, inorganic phosphate and pyrophosphate; OXA, oxaloacetate; α-KGA, α-ketoglutarate; Cr-P, creatine phosphate; Cr, creatine. Fig. 2 (right). "Flash activation" of glycogen phosphorylase by Ca^{2+} and ATP as observed in glycogen particles isolated from rabbit muscle. Relative velocity (V) is plotted against time in minutes, complete activation occurring well within the first seconds of addition of Ca^{2+} and ATP. Adenosine triphosphate is required for phosphorylation of the phosphorylase b dimer to the phosphorylase a tetramer, a reaction catalyzed by phosphorylase kinase, shown in the lower panel. The Ca^{2+} serves to increase by about 15-fold this enzyme's affinity for its substrate, phosphorylase b. At the same time, Ca^{2+} and ATP are both required for inhibition of the reverse inactivating reaction, catalyzed by phosphorylase phosphatase and converting the a tetramer back to b dimer. [After Heilmeyer *et al.* (*16*)]

without limit during glycolytic activation. As a result, the lactate concentration can become very high and muscle tissue sustains higher lactate accumulations than any other tissue in the vertebrate body.

From the above considerations, we conclude that among the vertebrates muscle glycolysis is one of the most efficient anaerobic machines invented by nature. In its evolutionary development, selection seems to have favored two fundamental characteristics:

1) High enzyme concentrations and abundant quantities of storage substrate (glycogen).

2) Specific enzyme variants designed for (i) large percentage swings from low to high activity states (up to several hundredfold activation), (ii) exponential rates of change from low to high activity, and (iii) accumulation of large quantities of lactate.

The first characteristic gives muscle a high glycolytic potential; the second brings that high potential under tight metabolic control.

Muscle Glycolysis in Marine Mammals

The basic design of muscle glycolysis is so effective that when muscle of a diving mammal, such as the porpoise, is compared to that of an animal such as the common laboratory rat, or man, only a small number of modifications are observed, and these are all, in effect, elaborations on the theme already described. Thus, when one considers muscle enzyme concentrations within the glycolytic chain (*28*), the major differences between a marine mammal and the laboratory rat are (i) increased amounts of phosphoglucomutase, aldolase, α-glycerophosphate dehydrogenase, and lactate dehydrogenase, and (ii) decreased amounts of pyruvate kinase. A third notable difference is that a marine mammal has unusually high concentrations of fructose diphosphatase, a nonglycolytic enzyme nonetheless involved in glycolytic control (*29*). Also, preliminary evidence suggests an unusually fast phosphorylase activation (b $\rightarrow$ a conversion) in diving animals (*28*).

On closer examination, one set of observations can be readily explained. Thus, because muscle in marine mammals is cut off from blood circulation during diving, it must rely less on blood glucose and more on muscle glycogen than would muscle in a terrestrial mammal; this is reflected in a more sensitive phosphorylase control and an increased phosphoglucomutase content. Second, an active aldolase contributes to tight regulation of the concentration of fructose-1,6-diphosphate, a key regulatory metabolite in the overall pathway. Third, muscle cells in divers must be prevented from becoming highly reduced and this requirement is reflected in higher titers of lactate dehydrogenase and α-glycerophosphate dehydrogenase. The high fructose diphosphatase and low pyruvate kinase titers, however, are not as easily explained.

Function of Muscle Fructose Diphosphatase

In the degree to which muscle glycolysis can oscillate between low activity and high activity states (between basal and maximally activated states), this multienzyme pathway is undoubtedly unique in the vertebrate body. In a relatively sluggish animal, such as the frog, it is possible to demonstrate a 100-fold glycolytic activation in the sartorius muscle within seconds of electrical stimulation, and under extreme conditions a 600-fold activation can be obtained (*15*). In fast-swimming divers, such as the porpoise, one would anticipate at least a comparable capacity, both in terms of speed of response and in terms of percentage activation. Achieving such large absolute activation calls for an essentially "completely off–completely on" catalytic behavior at key control sites, such as at the fructose-6-phosphate $\rightleftarrows$ fructose-1,6-diphosphate interconversion (*29*). How this is achieved is not yet clear, because the concentration of no single regulatory metabolite changes by a large enough factor to account for the degree of activation observed. One possibility is that various metabolites act in a synergistic manner to bring about the required on-off behavior; to some extent, this certainly occurs, but the highest estimates of the degree of phosphofructokinase activation that can be achieved by such means range between 200-fold and about 350-fold (*20*). In obtaining these estimates, a coordinated time course of change in the concentrations of various regulatory metabolites is assumed; if there is any lag or "slop" in the system (that is, changes in positive and negative modulator levels being slightly out of balance) these high values become greatly reduced. Even if these estimates are accepted as physiologically relevant, they are still well below the potential requirements for phosphofructokinase activation in fast-swimming divers such as the porpoise, in "burst" running of certain terrestrial animals, or in "burst" flight patterns of birds such as the pheasant (*29*). Newsholme (*29*) has argued that what is required to account for the on-off behavior at sites such as the fructose-6-phosphate $\rightleftarrows$ fructose-1,6-diphosphate interconversion is some sort of mechanism for amplifying metabolic "signals." Of a number of possibilities, muscle glycolysis seems to rely most on a fructose diphosphatase–based amplification mechanism.

In physiological terms, fructose diphosphatase catalyzes a reaction which is the reverse of that catalyzed by phosphofructokinase. Although phosphofructokinase is regulated by a large number of modulators, fructose diphosphatase seems to be under the regulation of only one effector compound: AMP. Adenosine monophosphate is a potent inhibitor of all fructose diphosphatases thus far examined, except for than in bumble bee flight muscle, where the enzyme is involved in a thermogenic function (*30*). When fructose diphosphatase was first discovered in muscle its function was unknown, and its presence posed a perplexing problem, since the simultaneous function of both enzymes in the same cell sets up a futile carbon cycle and a net ATP hydrolysis. That perhaps is precisely the point. Since both phosphofructokinase and fructose diphosphatase respond (in opposite manner) to at least one signal in common (AMP), the sensitivity of the overall fructose-6-phosphate $\rightarrow$ fructose-1,6-diphosphate conversion will be far greater than if only phosphofructokinase (or only fructose diphosphatase) were AMP-sensitive, or if only phosphofructokinase were present in the cell. That is, a single signal aimed at two such oppositely directed reactions is greatly amplified if one enzyme is activated while the other is inhibited by that signal. The degree of amplification depends on the ratio of the rate of the cycling to the rate of glycolytic flux, and in muscle the rate of cycling will, of course, dramatically depend on the fructose diphosphatase activity. This situation seems important enough to illustrate with a set of simple calculations [taken from (*29*)].

Consider two muscle types, one with low (0.1 arbitrary unit of) fructose diphosphatase activity, the other with high (5.0 units of) fructose diphosphatase activity. Assume in both a basal resting fructose-6-phosphate $\rightarrow$ fructose-1,6-diphosphate flux rate of 0.1 unit. In

the case of the muscle with low fructose diphosphatase activity, a basal flux rate of 0.1 unit depends on 0.2 phosphofructokinase unit minus 0.1 fructose diphosphatase unit. Now a twofold change in the AMP concentration doubles the phosphofructokinase activity while simultaneously halving the fructose diphosphatase activity; the activated phosphofructokinase rate is 0.4 unit (0.2×2) while the inhibited fructose diphosphatase rate is 0.05 (0.1/2). The net conversion rate (fructose-6-phosphate → fructose-1,6-diphosphate) is now 0.35 unit ($0.4 - 0.05$), representing a 350 percent increase. In the high fructose diphosphatase case, a basal flux rate of 0.1 unit depends on 5.1 phosphofructokinase units minus 5.0 fructose diphosphatase units. Assume that a twofold change in AMP concentration also leads to a doubling of phosphofructokinase activity and a halving of fructose diphosphatase activity; the activated phosphofructokinase rate becomes 10.2 units, the inhibited fructose diphosphatase rate becomes 2.5 units, and the overall activated (fructose-6-phosphate → fructose-1,6-diphosphate) flux rate is 7.7 units. Compared to the basal rate of 0.1 unit, this represents a 7700 percent change in net fructose-6-phosphate → fructose-1,6-diphosphate conversion.

Clearly, for a given muscle phosphofructokinase content, the higher the fructose diphosphatase, the greater the amplification of a given AMP signal. In this context, our observation (*28*) that the ratio of fructose diphosphatase to phosphofructokinase in porpoise muscle is about two to three times higher than in frog sartorius muscle, or in a variety of other mammalian and bird muscles, can be fully appreciated.

Regulatory Nature of Muscle Pyruvate Kinase in Diving Mammals

An explanation for the comparatively low pyruvate kinase content of porpoise muscle had to await detailed kinetic studies of the enzyme. Others had observed that, when divers are treated as a single group, the pyruvate kinase content of muscle correlates with the length of diving each species is capable of achieving (*31*), and such a correlation between glycolytic capacity and pyruvate kinase titer in fact also holds for normal mammalian tissues (*32*). However, within diving animals as a group pyruvate kinase concentrations are lower than in the common laboratory rat (*14, 28, 31*). What diving animals seem to have done at this locus is to accept an overall reduction in the activity of pyruvate kinase in favor of designing an enzyme which is much more sensitive to metabolite regulation. Unlike the enzyme in terrestrial mammals, muscle pyruvate kinase in diving vertebrates is under tight feedback inhibition by ATP, alanine, and probably citrate (*33*), and under strong feedforward activation by fructose-1,6-diphosphate, which returns the pyruvate kinase maximum potential to the high range expected for highly active glycolysis. Moreover, as with other regulatory pyruvate kinases, fructose-1,6-diphosphate not only directly activates the enzyme (by affecting both the Michaelis constant, K_m, and the maximum velocity of the enzyme-catalyzed reaction) but also strongly reverses the inhibitory effects of negative modulators. These regulatory characteristics are, in fact, commonly observed in muscle pyruvate kinases of lower animals (*24, 34*), but appear to have been lost in most mammals. Diving animals have retained (or regained) this tight control over pyruvate kinase in part because of a high reliance on glycolysis during diving, but mainly because of important control requirements imposed on muscle at the end of the dive—that is, during the anaerobic-aerobic transition, when muscle metabolism switches from glycogen to other fuels. These requirements arise from the fact that when the animal is not diving, the preferred fuel is fat, and standard *RQ* values are about 0.7 for most marine mammals thus far studied (*2*). To put it more accurately, then, an important consequence of the diving habit is a metabolic organization that can oscillate efficiently between glycogen catabolism (during anaerobic portions of the dive) and fat catabolism (during steady state aerobic work, such as surface swimming, migrations, and so forth). In considering how this oscillatory behavior is controlled it is convenient to look at the problem in terms of the anaerobic-aerobic transition and vice versa.

How Is Lipid Spared during Anaerobic Glycogenolysis?

The problem of sparing lipid during anaerobic metabolism is of course not unique to diving mammals. In better studied species, it is well established that fatty acid oxidation is "turned off" during anaerobic glycolysis, but to date only one metabolite signal—lactate—has been found to play a role in integrating these events (*17*). Thus, increasing lactate concentrations serve to inhibit the mobilization of triglyceride by inhibiting the first step in fat catabolism, that catalyzed by lipase. In addition, the mobilization of adipose triglyceride is under hormonal control, and this may contribute to the required integration between triglyceride and glycogen metabolism. Although neither mechanism has been studied in detail in any marine mammal, it is tempting to speculate that aerobic triglyceride catabolism in red and intermediate type fibers of the marine mammal is under hormonal control by direct effects on the high lipase content of these muscles (*5*). During prolonged diving, when blood flow to the muscle is shut off, such hormonal signals also would be automatically turned off, leading to a drop in the ratio of active to inactive lipase. At the same time, rising lactate concentrations would serve to directly inhibit any lipase activity remaining, thus contributing to an effective sparing of lipid at a time when glycogen fermentation is favored. At the end of the dive, when O_2 is abundant, the diving mammal is faced with the opposite problem of turning off glycolysis at the same time that it is activating fat metabolism.

How Is Glycogen Spared during Aerobic Periods?

Fortunately, the answer to this question is better understood. In mammals in general, it is now clear that during the anaerobic-aerobic transition, when fatty acid oxidation is initiated, a number of profound fluctuations in various Krebs cycle intermediates occur. Of these, the percentage increase in citrate concentrations is unusually high and this metabolite serves as a feedback inhibitor of phosphofructokinase, effectively blocking glycolysis when that block is appropriate (*12*). The same "information channel" is used in various tissues of the vertebrate, but as we have pointed out, muscle phosphofructokinase is unusually sensitive to citrate (about ten times more so than, for example, the liver isozyme), and thus in muscle this control interaction is perhaps most effective. As far as we know, the same control mechanism at this locus in glycolysis also operates in tissues of diving vertebrates (*35*), but whereas this seems to be a sufficient

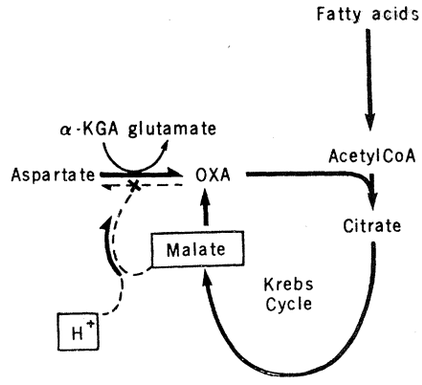

Fig. 3. Favored direction of aspartate aminotransferase function during anaerobic-aerobic transition in diving vertebrates such as the porpoise. The aspartate affinity of the mitochondrial enzyme is about fivefold higher than that of the cytoplasmic isozyme, and it therefore competes effectively for intramitochondrial aspartate. Its K_m for oxaloacetate is about tenfold higher than intramitochondrial oxaloacetate concentrations, a factor that also favors function in the direction of oxaloacetate production. Moreover, malate concentrations are increasing at this time, due to Krebs cycle activation, and malate serves as a potent inhibitor of the backward reaction. A low *p*H occurring at the end of the dive (*43*) further potentiates the malate control at this locus. [Data from Owen and Hochachka (*37*)]

mechanism in muscles of typical terrestrial mammals, it is by no means the only way to turn off glycolysis during aerobic periods in diving vertebrates. In vertebrate divers, pyruvate kinase is also an allosteric enzyme, also highly sensitive to citrate, and this would supply an additional means for blocking glycolysis during periods of fat catabolism (*33*). A third and most important mechanism for blocking glycolysis at this time involves alanine inhibition of pyruvate kinase, but a full appreciation of the physiological significance of this effect requires a brief digression into the nature of Krebs cycle activation during fatty acid oxidation.

It is of course important to realize that the "end product" of the β-oxidation spiral is acetyl coenzyme A, which is channeled into the Krebs cycle at the citrate synthase–catalyzed reaction. In the rat, during the anaerobic-aerobic transition, activation of the β-oxidation spiral leads to a momentary piling up of acetyl coenzyme A, as the oxaloacetate reserves for citrate synthesis are inadequate (*36*). In a diving animal such a limitation could be crippling. Thus, the diving habit leads in muscle to another important metabolic requirement: an efficient source of oxaloacetate that can be turned on during fatty acid oxidation and Krebs cycle activation. The source of oxaloacetate is aspartate via the aspartate aminotransferase step. In marine mammals, such as the porpoise, the activity of this enzyme in muscle is up to 17 times higher than in terrestrial species (*37*), and its catalytic properties are such as to favor oxaloacetate production in the mitochondria during the anaerobic-aerobic transition (Fig. 3). Glutamate, produced in the reaction, in turn transaminates with pyruvate to regenerate α-ketoglutarate; this process, catalyzed by alanine aminotransferase, which also occurs in unusually high amounts in muscle of marine mammals (*37*), leads to alanine accumulation (*38*). The total amount of alanine accumulated under such conditions is equal to the summed increase in concentration of all Krebs cycle intermediates (*36*). That is a fundamental insight, for it emphasizes that alanine is perhaps the single best metabolite signal of the degree to which the Krebs cycle is activated. It is therefore not surprising that alanine is such a good inhibitor of pyruvate kinase in diving vertebrates (*33*), for the greater the degree of Krebs cycle activation, the greater the degree to which pyruvate kinase is blocked by alanine and carbohydrate reserves are spared for anaerobic excursions.

Anoxia Tolerance of Diving Turtles

The kinds of molecular adaptations seen in diving mammals seem to reach their zenith in diving turtles, such as the green sea turtle (*Chelonia mydas*) and the red-eared turtle (*Pseudemys scripta*), which are capable of diving for many hours at a time or, in some circumstances, for days at a time. These organisms come closer to becoming "facultative anaerobes" than any other vertebrate group, and it is therefore instructive to examine them in some detail.

Diving turtles as a group are several times more tolerant of anoxia than other reptiles and far more tolerant of anoxia than mammals. The green sea turtle, for example, can readily dive for at least two to several hours at a time (*38*) and is reputed to spend "resting" periods of several hours under water. The red-eared turtle survives dives as long as 2 weeks at 16° to 18°C despite a total lack of electron transport mediated O_2 consumption. During such anoxic dives of the red-eared turtle, blood glucose rises from 3 to about 60 m*M*, blood lactate rises to over 100 m*M*, and blood *p*H drops from 7.9 to 6.8 (*33*, *35*). In the green sea turtle, following a 2-hour anoxic dive, blood lactate concentrations rise to above 40 m*M* (*38*). Jackson (*8*) has pointed out that after the first hour or so in a normal experimental "dive" where the animal is fairly quiescent, all blood and tissue O_2 stores are fully depleted; yet the animal of course can continue its dive for many more hours on a glycogen-based fermentation whose measured capacity in caloric terms is about 15 to 20 percent of that of the animal's aerobic metabolism. Under such extreme diving conditions, all the tissues of the body must be able to maintain their functions in anoxia. Since the vertebrate heart and central nervous system typically display an absolute dependence on O_2, special attention has been focused on the heart of the diving turtle as an ideal vertebrate organ in which to sort out evolutionary mechanisms of anoxia adaptation.

The Anoxic Turtle Heart

Although the picture here is not yet complete, current information already confirms our observation that, in compensating for the temporary unavailability of O_2, vertebrate divers activate anaerobic ATP-yielding capacities by (i) increasing the glycolytic potential and (ii) modifying regulatory properties of key enzymes to keep that high potential under tight control. In diving turtles, the high glycolytic potential is seen in both glycogen and enzyme concentrations. The cardiac glycogen content is ten times higher in the turtle than in terrestrial mammals and some four to five times higher than in diving mammals such as the seal (*9*). This feature in itself creates a higher glycolytic potential, and it is bolstered by high quantities of glycolytic enzymes (*39*). Their integrated function is so efficient that transient changes in ATP/ADP ratios are not readily detectable during aerobic-anaerobic transitions (*40*). Whereas a drop in the ATP concentration is an important means of deinhibiting phosphofructokinase and thus turning on glycolysis in the rat heart during the aerobic-anaerobic transition, it is not a useful signal in the heart of a diving turtle; not surprisingly, turtle heart phosphofructokinase is remarkably insensitive to ATP (*35*). The usual regulatory role of ATP in

this tissue is taken over by creatine phosphate, which is known to be rapidly depleted in anoxia (*41*), and which therefore supplies the tissue with a good measure of glycolytic requirements. Under aerobic conditions, creatine phosphate concentrations are high and serve to keep glycolytic rates low by potent phosphofructokinase inhibition. In the absence of any other metabolic controls, a drop in creatine phosphate, initiated by NADH activation of creatine kinase (*42*), would serve to deinhibit phosphofructokinase and activate glycolysis. This deinhibition, moreover, is greatly potentiated by fructose-1,6-diphosphate, one of the products of the phosphofructokinase reaction. As fructose-1,6-diphosphate is known to momentarily accumulate during glycolytic activation, it leads to a further phosphofructokinase activation and thus to an autocatalytic increase in the rate of its own production (Fig. 4).

Fructose-1,6-diphosphate control of glycolysis in the diving turtle is particularly pivotal because, in addition to the effects on phosphofructokinase described above, it plays a key role in integrating phosphofructokinase activity with that of pyruvate kinase, the next major control site in the glycolytic pathway (Fig. 4). Fructose-1,6-diphosphate control of pyruvate kinase is of two forms: first, it directly activates the enzyme by greatly increasing enzyme-substrate affinity, and second, it reverses and overrides inhibition of the enzyme by a variety of metabolites, ATP, alanine, and citrate probably being the most important. These control interactions are sufficient (i) to account for an exponential rate of change of pyruvate kinase activity during the aerobic-anaerobic transition, and (ii) to closely integrate the activities of phosphofructokinase and pyruvate kinase, both major control sites in the glycolytic path.

In tissues such as the heart, which even in the diving turtle is supplied for as long as possible with oxygenated blood, important adjustments in lactate tolerance have occurred, in part through altered *p*H optima of glycolytic enzymes (*33, 35*), in part through improved buffering capacity (*43*), and in part through evolutionary changes in heart-type lactate dehydrogenase. Heart lactate dehydrogenase is usually strongly substrate-inhibited so that whenever pyruvate concentrations rise, lactate dehydrogenase is inhibited and pyruvate channeling into the Krebs cycle is in effect favored; this characteristic is missing in heart lactate dehydrogenase of turtles as well as other diving species (*44*). Equally important, the enzyme affinity for pyruvate decreases to the same range as in muscle. In other words, the kinetic properties of turtle heart lactate dehydrogenase have been tailored to resemble those of the muscle isozyme, presumably because of the increased reliance of the heart on anaerobic metabolism.

Alpha-glycerophosphate dehydrogenase, also present in the turtle heart, has not yet been kinetically characterized (*45*). Together with lactate dehydrogenase, it presumably functions to maintain the unusually high cytosolic NAD/NADH ratios that occur in the turtle before diving (*46*). Even after 6 to 12 hours of anoxia, turtle tissues retain a higher oxidation potential than homologous rat tissues under normal aerobic conditons (*46, 47*). Not surprisingly, the isolated turtle heart can sustain a four to five times greater contractile work load than can the rat heart under identical anoxic conditions (*48*).

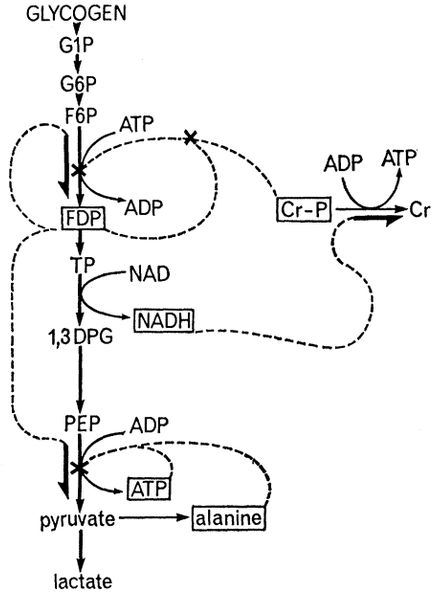

Fig. 4. The diving turtle: control circuitry in glycolysis of the heart. Activation is indicated by dark arrows, inhibition by dark crosses. A major modification in this control setup is the replacement of ATP inhibition of phosphofructokinase by creatine phosphate inhibition. Activation of creatine kinase by NADH integrates creatine kinase activation with glycolytic activation. Fructose-1,6-diphosphate takes on a particularly pivotal role in that it (i) reverses creatine phosphate inhibition, (ii) is a product of the phosphofructokinase reaction which activates phosphofructokinase and (iii) activates pyruvate kinase by a feedforward mechanism. [Data from (*33, 35, 42*)]

Long-Term Anaerobic Capacity

That the kinds of metabolic modifications we are discussing lead to the development of a long-term anaerobic potential has been implicit to this point. To reiterate, however, the three most important characteristics allowing extended anaerobic function include (i) the maintenance of high glycogen concentrations for anaerobic use; (ii) the tolerance of a high lactate accumulation, through more efficient buffering, more acidic *p*H optima of glycolytic enzymes, and an effective means for lactate removal as well as its subsequent metabolism after the dive; and (iii) the high NAD/NADH ratio that characterizes the animal before diving. Whereas it is worth emphasizing that these features would be useful in any tissue within the body and probably are somewhat accentuated in all tissues of the diving animal, no discussion of the long-term capacity of diving animals would be complete without a brief consideration of metabolic events occurring in the central circulation.

Potential for Cycling Metabolites in the "Heart-Lung-Brain Machine"

Although a premium is placed on unusually large glycogen depots in the heart and brain of divers, it is clear that, at least in the turtle, glucose remains an important source of carbon and energy for the brain during prolonged diving (*41*). This is an important insight, for it indicates that any lactate formed in these tissues can be transported to the liver in exchange for glucose coming from the liver. That is, the Cori cycle remains functional, and even in anoxia these tissues need not accumulate large quantities of lactate. Perhaps that is why lactate concentrations in the central circulation of diving animals typically do not rise by more than 10 to 20 percent during the dive (*1, 2*), but of course shoot up at the end of the dive when blood circulation to peripheral tissues is opened.

Furthermore, other metabolites involved in different metabolic functions may also be cycled between tissues served by the central circulation. A possible example of this is related to redox regulation. A characteristic of the diving animal is its ability to sustain a relatively high NAD/NADH ratio in the face of prolonged anoxia. This appears to be true of several turtle tissues (*46*) as well as of the duck brain (*49*).

But how can this be achieved without large lactate accumulations in the tissue? One interesting possibility involves the transport of lactate, formed in the heart or brain, to the lungs. Because the lungs remain oxygenated even in severe hypoxia (*50*), conditions there are suitable for rapid oxidation of lactate back to pyruvate, which could be returned to the heart and brain for NADH oxidation and reconversion to lactate. Such an intertissue cycling of lactate and pyruvate would readily explain how the redox balance in heart and brain can be maintained in prolonged anoxia without a large lactate accumulation in the tissue or the blood.

Finally, extraglycolytic sources of energy may also contribute to long-term anaerobiosis. Chief candidates for this function are energy-yielding mitochondrial reactions. The substrate-level phosphorylation occurring during conversion of succinyl coenzyme A to succinate is one such reaction in the Krebs cycle (*34*); another, the oxidation of NADH by fumarate, is of special interest since it includes a phosphorylation at the first site of the respiratory chain. This "fumarate reductase" reaction, although functional in a variety of mammalian tissues, occurs in highest activity in the heart (*51*). Succinate formed by it is thought to be transported to the lungs where it is reoxidized to fumarate and malate, which in turn are recycled back to the heart. The metabolic significance of this reaction appears to be in the uptake of Ca^{2+}, which is essential for cardiac contractility; thus, Ca^{2+} uptake is not promoted by glycolysis in the anoxic heart but is stimulated by fumarate (*50*). The energy requirements of Ca^{2+} transport are low compared to those of muscle work per se (supported by glycolysis), and it is therefore not surprising that blood succinate concentrations after diving are substantially lower than blood lactate concentrations (*38*). If these considerations are correct, they indicate that (i) succinate, fumate, and malate act as an auxiliary electron shuttle between the heart (or other tissues) and the lung, and (ii) their metabolism sustains critical processes that are not supported by glycolysis. As with the concept of pyruvate-lactate cycling between the lungs and heart (or brain), the overall process takes on an intertissue, cyclic, and catalytic function that might greatly extend the time period of breath-hold diving without leading to large net accumulations or net depletions of these key intermediates.

Human Diving

In the last decade or so it has become apparent that the human diver displays many of the physiological adjustments common to animal divers. For example, the ama, diving women of Korea and Japan, display respiratory adjustments on a small scale that are not unlike those of the highly evolved diving vertebrates (*52*). Although humans do not dive for more than about 2 to 3 minutes, they sustain a fairly potent bradycardia, heart rates commonly decreasing as much as 50 percent within the first minute of a dive (*53, 54*). Such bradycardia is characteristic of the pearl divers of the Torres Strait archipelago, who are conditioned over many years for underwater work (*53*). The trained Caucasian diver, on the other hand, appears to increase his heart rate about twofold just before diving; during the dive the heart rate then falls steadily, usually becoming significantly lower than in the predive state (*54*). In addition, peripheral tissues, particularly skeletal muscles, appear to be cut off from the general circulation by peripheral vasoconstrictions similar to those observed in animal divers (*55*). Such peripheral vasoconstriction occurs in the conditioned pearl diver as well as in the novice (*53, 55*). Not surprisingly, Scholander *et al.* (*53*) found that lactate, produced in the muscle of pearl divers during the dive, is stored in the tissue until the end of the dive; then as the blood circulation to the muscle is reopened, lactate is released into the blood at concentrations (after dives of 1 to 2 minutes) of maximally 3 to 4 m*M*. Similar results have been obtained with Caucasian divers (*56*), and on the basis of these studies it is widely accepted that man has the basic physiological machinery for expressing the so-called diving syndrome. But what of the cellular, metabolic adjustments that are called for as a consequence of routine diving? To what extent are these expressed in divers such as the amas, who dive between 30 and 60 times an hour for several hours at a stretch?

Unfortunately, the data here are scarce. Yet, if there is a single thought emerging from our analysis of diving animals, it is that both the amount and the kind of regulatory enzymes present at key metabolic loci have been tailored in diving animals to facilitate an efficient oscillation between glycogen-based fermentation (during the dive) and fast-based aerobic metabolism (after the dive). In man, conditioned for diving over short time periods, this kind of biochemical adaptation presumably would not be genetically fixed, except perhaps in the amas, who are known to have sustained their life-style for at least 1500 years (*52*). The requisite functional organization of metabolism, however, might be achieved by regulating the proportions of different isozymes at each of the key loci involved. Phosphofructokinase, for example, occurs in multimolecular forms, the B form being highly insensitive to creatine phosphate and to citrate but highly sensitive to ATP inhibition, the A form showing the reverse sensitivities (*23*). Similarly, isozymic forms of pyruvate kinase vary greatly in sensitivity to ATP and alanine (*33, 34*); adjustment of their relative concentrations in tissue could greatly alter the control of carbon flux through that particular locus in metabolism and significantly alter that tissue's capacity to oscillate between anaerobic and aerobic metabolism. Indeed, the isozymic differences in phosphofructokinase, pyruvate kinase, and lactate dehydrogenase between tissues within the individual appear to be notably similar to the differences between diving and nondiving vertebrates, and this may prove to be a basis for the evolutionary development of the enzyme forms favored in diving animals.

Summary

The basic theme of our analysis of metabolic consequences of diving is that muscle glycolysis, even in the laboratory rat, already is a most impressive anaerobic machine, and further improvement of its capacity and efficiency in muscle of diving vertebrates seems to have involved only a modest number of modifications. Thus, the steady state concentrations of a few glycolytic enzymes are increased, reflecting a higher overall glycolytic potential and an improved capacity to maintain NAD/NADH ratios in anoxic stress. To retain control of the higher glycolytic capacity, at least two additional modifications are now known: (i) muscle fructose diphosphatase activity in divers is one of the highest thus far reported for any animal species, the enzyme appearing to function in amplifying the AMP signal for glycolytic activation, and (ii) muscle pyruvate kinase, although having a lower specific activity, occurs as a regulatory

enzyme, highly sensitive to feedforward activation by fructose-1,6-diphosphate and feedback inhibition by ATP, alanine, and citrate. The fructose-1,6-diphosphate feedforward activation presumably functions during the aerobic-anaerobic transition in the dive, while the feedback inhibitions by ATP, alanine, and probably citrate (all acting in effect as end products of aerobic, fatty acid catabolism) appear to function during the anaerobic-aerobic transition at the end of diving. The latter characteristic emphasizes another important consequence of the diving habit: a metabolic organization that swings between an anaerobic, glycogen-based fermentation and an aerobic, fat-based oxidative metabolism. The control requirements imposed on muscle by this metabolic organization have led to the appearance of unusually high titers of asparate and alanine aminotransferases. The mitochondrial form of aspartate aminotransferase is designed to spark the Krebs cycle by increasing the availability of oxaloacetate at the same time that acetyl coenzyme A is being produced by β-oxidation. Alanine aminotransferase regenerates the α-ketoglutarate required for this process and leads to the accumulation of alanine, which plays a key role in turning off glycolysis at this time.

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