METABOLIC CONSEQUENCES OF DIVING: THE ANOXIC TURTLE

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B.Sc. (Hons.), University of Calgary, 1971

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department

of

ZOOLOGY

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

July, 1974

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Date June 24, 1974

ABSTRACT

Catalytic and regulatory properties of phosphofructokinase (PFK) (EC 2.7.1.11), pyruvate kinase (PK) (EC 2.7.1.40), and creatine kinase (CK) (EC 2.7.3.2) from the heart of the red-eared turtle (Pseudemys scripta elegans) were studied. Particular attention was given to those properties of the enzymes which could help to explain the high glycolytic efficiency in this tissue and so provide insights into the selective forces involved in the evolutionary development of an extreme tolerance to anoxia.

The control of glycolytic flux in turtle ventrical muscle has been vested primarily in phosphofructokinase and pyruvate kinase. Creatine phosphate and fructose diphosphate play pivotal roles in the channelling of carbon through the pathway and in the production of metabolic energy as ATP. The levels of these key metabolites are in turn tightly regulated. One of the enzymes studied (phosphofructokinase) controls the levels of fructose diphosphate produced and creatine kinase (itself modulated by the cell's redox balance) controls creatine phosphate levels.

When oxygen levels are reduced, NADH accumulates because of a decrease in electron transport chain regeneration of NAD (129). This leads to an effective activation of creatine kinase by lowering the K_m for creatine phosphate. This activation causes a drop in creatine phosphate levels without the decline in ATP levels that are seen at the onset of hypoxia in other tissues (4). Since the high levels of creatine phosphate present in aerobic heart are responsible for the inhibition of phosphofructokinase by greatly increasing the K_m for its substrate, fructose-6-phosphate, this drop in concentration deinhibits the enzyme and leads to a flush of its product, fructose diphosphate. The increase in fructose diphosphate

(1) serves to activate phosphofructokinase by itself, and (2) further reduces the effect of creatine phosphate by a deinhibition of the enzyme. These two effects cause an autocatalytic increase in flux through this locus. Fructose diphosphate also feed-forward activates pyruvate kinase by decreasing the K_m for its substrate, phosphoenolpyruvate, and serves to deinhibit this enzyme which is normally inactive due to alanine and ATP inhibition.

The most important feature of such a regulatory system is that it is a kind of autocatalytic cascade. Once the activation of creatine kinase is initiated by the redox imbalance inherent in anaerobiosis, all the various regulatory interactions potentiate one another. Drops in inhibitor levels lead to increases in activator levels and these activators serve to further deinhibit. These interacting effects serve to potentiate anoxic production of energy to compensate for the temporary depletion of oxygen in diving stress and in long periods of hibernation in this turtle, Nature's premier vertebrate anaerobe.

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ACKNOWLEDGEMENTS

More than thanks are due to my supervisor, Dr. Peter Hochachka, who took me in out of the storm and completely reshaped my scientific future. I would like to take this opportunity to express my deep appreciation to him for his invaluable advice, encouragement and support throughout the course of this work.

Thanks are due to the many helpful people who kept me going both personally and scientifically. Also to S. ball and all those associated for keeping Science in perspective.

Special thanks are due to J. Collicutt for understanding during the dog-days of thesis writing and to H. Lakusta for encouragement and moral support throughout my studies.

Dedicated to my family.

CHAPTER I:

Introduction

"Environmental adaptations" (or, the structural and functional characteristics of an organism that seem designed to enhance its chance for success in its particular habitat) have been charted at all levels of biological organization: behavior patterns, anatomical pecularities and physiological function. Adaptations to diving and low oxygen tensions in nature are evident at each organizational level: behaviorally (some animals dive and/or live at the bottom of the ocean while we are stuck here); anatomically (good divers are streamlined and have limbs adapted for pushing water); physiologically (the diving syndrome, which allows longer and so more productive dives); and last but not least, biochemically. Adaptations at the biochemical level are the least well understood and appreciated, probably due to the fact that these changes are adaptive at the level of basic metabolic function and are not apparent macroscopically. Deep inside its cells a diving animal is not just a laboratory rat in disguise but contains a metabolic organization as peculiar to its habits as its flippers.

Species with highly developed anaerobic capacities are to be found among several invertebrate groups including the Mollusca, Arthropoda and Echinoderma (1). Less well developed but nonetheless impressive anaerobic capacities have appeared amongst the vertebrates. Benthic fishes have been captured in the deep waters of Lake Tanganyika, despite the fact that the lake is anoxic at all depths below the thermocline (2). Similarly, the Crucian carp in Europe often becomes ice-locked in small ponds, sloughs, and lakes. As the winter progresses, many of these bodies of water become depleted of O_2. The carp survives 2-3 month periods of such imposed anoxia with no apparent ill effects (3). Diving marine reptiles, birds, and mammals also rely heavily upon anaerobiosis (4,5,6).

At the biochemical level there are 2 strategies of adaptation to low oxygen availability (7):

1. Exploitative: Intertidal bivalves among others are able to invade anoxic environments for extended or indefinite periods of time. This strategy does not depend upon a return to oxygen and involves (8) (a) deleting metabolic dead ends, notably lactate, (b) modifying the kinetic properties of key branchpoint enzymes to allow an efficient aerobic to anaerobic transition, e.g. the PEP branchpoint - the 2 enzymes metabolizing PEP are set up so that they are not able to function at significant rates simultaneously. With the onset of anaerobiosis the intracellular pH of the adductor muscle drops and this, coupled with a hydrogen-ion potentiated end product inhibition by alanine, allows the carbon flow - originally toward the Krebs cycle and oxidation - to be diverted to alternate end products (especially succinate) with the concomitant production of extra ATP that would not have been otherwise available. (c) The coupling of other substrate level phosphorylations to the glycolytic reactions, thus increasing the potential yield of high energy compounds. This is achieved in the oyster by the stoichiometric coupling of carbohydrate and amino acid metabolism; glutamate is utilized along with glucose units. This coupling principle is also used in vertebrates to supplant glycolysis in anaerobic tissues such as the kidney (9).

The second strategy, 2. Compensatory: Some organisms (including the diving vertebrates) compensate for the temporary depletion of oxygen by activating high capacities for glycolytic generation of ATP. This biochemical strategy depends upon the ultimate return to aerobic conditions, and the metabolic adaptations occurring are typified by vertebrate white

muscle. Under a variety of conditions, including the "burst" activities of running and diving, the rate of oxygen delivery does not meet the total demands of all the tissues. The vertebrate solution is to cut off those tissues that have capacities to work in the absence of oxygen - white skeletal muscle in particular (10).

But before "diving" into the biochemical adaptations of muscles we should briefly dwell on the physiological adaptations of divers because, in general, biochemical adaptations are a last resort for the organism because they require at least some rearrangement of the fundamental chemistry of the cell (7,11).

The central feature of apnoeic diving is the cessation of external respiration during the period of submersion, with a gradual development of hypoxia and hypercapnia. The adaptation of the organism to sustain long periods of apnoea may be the result of several factors acting singly or in concert: an increased resistance to asphixia, increased oxygen stores in the body, and mechanisms allowing conservation of the limited stores of oxygen (12).

The conservation of oxygen during a dive takes several forms: the metabolic rate may be lowered (14), oxygen storage is augmented in aquatic mammals by increased quantities of circulating hemoglobin and muscle myoglobin, thus providing for general increased oxygen capacity (13). Also, these animals have a larger volume of blood and an increased Bohr effect. The goal of oxygen conservation is also aided by control mechanisms which allow the cardiac output to be selectively distributed to those tissues that need it the most. Oxygen available in circulating blood is thereby conserved and not squandered by the perfusion of tissues able to get by on a reduced supply

of oxygen throughout the dive (10).

These latter mechanisms involve cardiovascular changes which include bradycardia, a large decrease in cardiac output, and extensive peripheral vasoconstriction, which, in effect, reduces the diver to a "heart-lung-brain" machine. These changes are generally reflex in nature and are known as the diving syndrome. In most diving vertebrates the length of the dive is largely determined by the rate of the utilization of the internal oxygen stores, pointing up the central importance of these mechanisms to a diving mode of life.

These internal blood shuntings are designed to keep the most oxygen rich blood in constant contact with the most oxygen dependent tissues, the CNS and the heart. However, if apnoea is experimentally extended to near maximal duration, hypoxemia reaches such levels that blood oxygen may be insufficient for sustaining the needs of even these well perfused organs (15). The heart itself responds to this lowered O_2 level with ventricular dilation and reduced myocardial contractility which serve to maintain stroke volume but reduce oxygen demand (16). But eventually even this is not enough as the dive proceeds and this normally "obligate aerobe" must derive its energy from anaerobic sources. Divers have an increased inherent anaerobic capacity which is due to metabolic alterations that have evolved to allow them to utilize fuels normally inaccessible to the aerobic tissues of non-divers.

Metabolic Fuels

The two major metabolic fuels used by mammals are: carbohydrate, whose utilization does not require oxygen's presence but whose energy yield is low, and fat, the high octane fuel, which is a much more efficient fuel to store

and use but which can only be used in the presence of oxygen (17). It is known that organisms and tissues within organisms differ in their ability to oxidixe lipids during rest and during work. Heart, red muscle and kidney cortex are tissues which are adapted for aerobic function and are known to oxidize lipids readily. White skeletal muscle on the other hand must rely on glycolysis during rapid bursts of activity. Other tissues, notably the CNS, are strictly dependent on respiration in most animals. Underlying a tissue's ability to burn a certain fuel and/or survive without oxygen are the metabolic adaptations peculiar to each tissue (18).

The diving animals are basically fat burners - in both heart and skeletal muscle fat is the preferred substrate, and so when oxygen stores begin to dwindle, each tissue must have mechanisms to make the switch-over from oxidative (fat) to anaerobic (carbohydrate) utilization. This will occur in the peripheral muscles of the diver first because these tissues are cut off from the circulation early in the dive and do not contain much in the way of an internal oxygen store. But eventually in the best divers, all the tissues must derive their energy needs anaerobically (19).

An important consequence then 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 divers, the oscillation between fat and glycogen based metabolism is accentuated and is seen to be under tighter metabolic control in these species than in non-divers (20).

This either/or utilization of fuel is accomplished by end product regulation of enzyme activity. Lactic acid, the terminal product of glycolysis,

inhibits the release of FFA into blood by adipose tissue. Thus, when the muscle is forced to rely on glycolysis due to low oxygen availability, the fuel reserves for aerobic metabolism, the lipid stores are spared. During aerobic metabolism when sufficient oxygen is present to support entry of acetylCoA residues into the Krebs cycle, citrate concentrations rise and feedback inhibit PFK, the major control valve in the glycolytic pathway; thus, under aerobic conditions the carbohydrate fuel reserves in the muscle are spared and glycolysis is dampened (21).

There are certain basic requirements and solutions to anaerobic stress that are used in Nature and these are typified by vertebrate white muscle. Nature has given us an excellent experimental anaerobic "machine" in muscle, which shows a wide selection of adaptations to anaerobic function. From a careful comparison of tissues such as liver (which does not depend critically upon glycolysis) with tissues such as white muscle (which during "burst activity" derives essentially all its useful energy from anaerobic mechanisms), it becomes clear that Nature "tunes up" the glycolytic machinery in several important ways (23).

When hypoxic conditions occur and the tissues convert to anaerobic glycolysis, 13 times the amount of glucose units are needed for the same ATP yield as the aerobic situation. There is a need therefore to maximize the glycolytic potential to compensate (19). 1) High substrate levels are needed to balance increased flux (4). 2) Muscle is seen to have higher levels of glycolytic enzymes than other less anaerobic tissues such as heart (23). 3) In muscle the metabolic rate must be able to swing quickly from low activity or fat repressed state to very high activity - a jump from 1% to 95% activity (24). This is accomplished by adjusting the "poise" of the system

with muscle specific forms of most of the glycolytic enzymes (25). 4) Part of maximizing the potential of the system is having efficient signals when to turn on and off. The calcium ions released on muscle contraction is the signal in muscle that links muscle contraction with biochemical events by activating phosphorylase (26). Another efficient mechanism is feed-forward activation, where a metabolite early in the pathway activates a limiting step later on, allowing increased carbon flow to proceed freely down the pathway (27). 5) Since glycolysis is activated, the system must allow for increased end product (lactate) accumulation. This is achieved by: buffering the acid produced by more concentrated buffers; elaborating control enzymes with more acidic pH optima (20); and by having a muscle specific LDH which has a 10-fold higher K_m for pyruvate to allow flux to proceed and not allowing the enzyme to saturate (28). This LDH is also insensitive to substrate inhibition. After long term anaerobic glycolytic function there also must be efficient ways of disposing of the lactate formed, ie,liver utilization of lactate via the Cori cycle (7).

These mechanisms in the vertebrate compensate for the temporary absence of O_2 by allowing for an impressive increase in the anaerobic ATP generating capacity. In the divers (red-eared turtle, marine mammals and diving birds), it is these "control" loci which one would expect to find even further adjusted for anaerobic function.

However, before looking at these control points in divers, it would be instructive to look at the control of glycolysis in general. This would help to pinpoint what to look for in the way of enzymatic adaptations and what experimental loci to probe.

Glycolytic Control

Recently it has become apparent that control of glycolysis never resides at one key site. Rather, the pathway is broken up into various segments, in each of which there appears to be a key control reaction; during operation, control is transferred from site to site along the pathway, but it is not yet certain of how this is organized in time (29). An initial approach to identifying the rate limiting and control factors of the glycolytic pathway is consideration of the maximal catalytic activities of the component enzymes under optimal experimental conditions. Reactions which have relatively low catalytic capacities represent the more probable sites for regulation of the overall flux of the pathway (30,31). Another method for locating control sites involves the application of the crossover theorem (32) to studies in which the levels of the intermediates of a pathway are measured during a transition between two steady states. Such perturbations have been induced by an aerobic to anaerobic transition (increased flux) in various tissues including rat heart (33,34) and turtle heart (35). A crossover is observed between F6P and FDP which indicates that the glycolytic flux is controlled primarily at the level of PFK.

An alternate approach to the location of rate-limiting steps is provided by a determination of those reactions which are far from equilibrium under in vivo conditions. Such reactions may be identified by calculation of an equilibrium constant from measurements of the levels of intermediates (the mass action ratio) and comparison with the apparent equilibrium constant determined in vitro under similar conditions. Penney and Shemerdiak (35) have found that in perfused turtle heart the mass action ratio of PFK is displaced from the apparent equilibrium constant showing that this enzyme catalyzes a reaction operating far from equilibrium. Comparisons of these

ratios in rat heart (36) as well as many other tissues (23) identifies PFK, glyceraldehyde-3-P dehydrogenase (G3PDH), PK and hexokinase (HK) as being far from equilibrium in vivo.

A role for glyceraldehyde-3-phosphate dehydrogenase in regulation of perfused rat heart during the increased glycolytic flux induced by an aerobic-anaerobic transition is shown by an early and transient rate limitation at this reaction (37). However, Rutter (38,39) has found five electrophoretically distinct bands of G3PDH activity in turtle heart which makes it unlikely that this enzyme is a control site. The major control points of energy metabolism in anoxic turtle heart are: phosphorylase (glycogenolysis), hexokinase, phosphofructokinase and pyruvate kinase.

Glycogen mobilization is initiated by glycogen phosphorylase, a regulatory enzyme par excellence. It is strategically situated between glycogen and the metabolic machinery required for its degradation, and is under the control of a variety of hormonal, ionic, and metabolite chemicals. Activation of glycogen phosphorylase can be triggered hormonally via the action of epinephrine, norepinephrine, glucagon, and other agents, and ionically by Ca^{++}. Both activators lead to the formation of an active tetramer, phosphorylase a, from an inactive dimer, phosphorylase b (18). The Ca^{++} activation appears to be of primary importance (particularly to diving vertebrates, where the blood flow and hence hormonal signals to muscle, are cut off during the dive) because it is thought to integrate metabolic events with mechanical (contractile) ones.

Reeves, 1963 (40) has shown that the turtle heart preferentially utilizes its large glycogen stores before switching to blood glucose as an energy source. This clearly implicates glycogen phosphorylase as a key

regulator of glycolysis during the initial phases of anoxia. Data comparing phosphorylase activity of various species demonstrate that while the turtle has less total enzyme than the mammalian hearts more of the enzyme is present in the active form (41). Turtle heart phosphorylase, unlike that of mammalian muscle, is not affected by the contraction cycle. G. Drummond (personal communication) found that turtle heart phosphorylase is too "labile" to get accurate measurements of its kinetic parameters. It is converted from the b to a forms too rapidly to get meaningful data. For this reason, as well as the fact that our lab is ill equipped to work on phosphorylase, this enzyme, although important, was not studied.

Phosphofructokinase

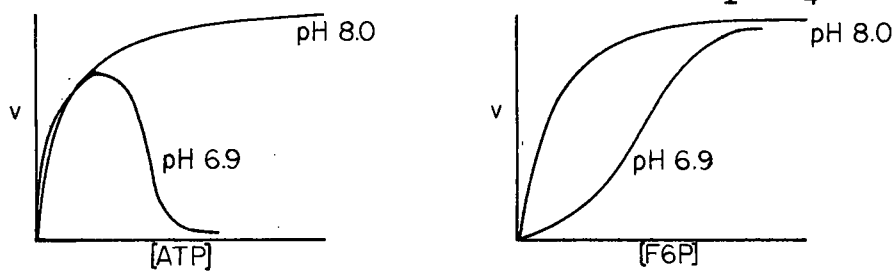
PFK is controlled *in vivo* by several different mechanisms (42) including: 1) aggregation $\rightleftharpoons$ disaggregation; 2) monomer $\rightleftharpoons$ polymerization (change activity); 3) bound and free forms; 4) ATP inhibited and ATP insensitive forms (43); 5) kinetic behavior. Enzyme and metabolite concentrations as well as pH affect the form the enzyme assumes and its relative activity. This situation may be somewhat simplified in turtle heart as when the enzyme is subjected to pH 5.8-6.5 it does become inactive but it cannot be reactivated again *in vitro* by substrates, activators, or increased enzyme concentration. When a special procedure is used (43) on turtle heart to isolate PFK aggregates, very little activity is obtained suggesting that perhaps in turtle heart aggregation-disaggregation effects are minimal and only the one form (MW 3.6×10^5) is active in glycolysis.

The problem of bound and free forms of PFK in heart has been investigated by Mansour (44) who found that only about 1/20 of the activity was obtained in a sheep heart high speed supernatant but if the pellet was incubated with

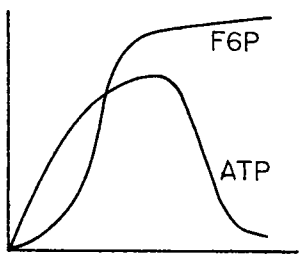
ATP and $MgSO_4$ the remaining activity was released. Performing identical experiments on turtle heart no particulate PFK was ever obtained and this level of control is also probably not important in the system under study.

ATP insensitive forms of PFK are obtained when the enzyme is purified with FDP and/or ATP present. The sensitivity is regained in most cases on dialysis and/or gel filtration but this does point to multiple in vivo forms of the enzyme depending on intracellular milieu.

The reaction catalyzed by PFK represents the first unique step in glycolysis; hence it is not surprising that the enzyme is profoundly regulated by various metabolites in a manner that controls rates of glycolysis in accord with the cells' need for energy or glycolytic intermediates (23,45-47). The complexity of the regulation of PFK lies in the fact that in addition to its multiple molecular forms it is regulated by a multiplicity of ligands. In typical PFKs both substrates and both products and pH are allosteric modifiers. Activators of PFK include AMP, F6P, FDP, P_i, NH_4^+ (23,48,49)

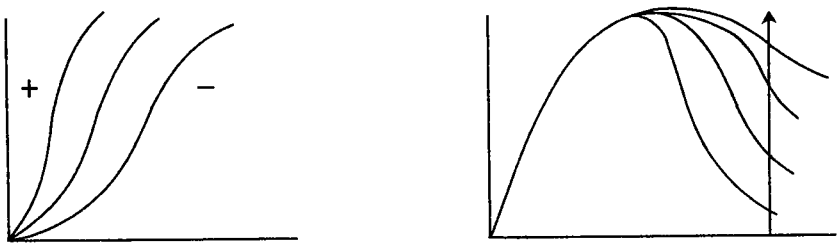


whereas ATP, citrate and creatine phosphate are inhibitors (50). ATP generally decreases the affinity of the enzyme for the second substrate F6P. In the presence of inhibitory concentrations of ATP, increasing the concentration of F6P has a cooperative effect on the activity of the enzyme.



Sigmoidal F6P curves mean that there are 2 sites of binding for this substrate - a high affinity site and a low affinity one. Binding of F6P at the first helps binding at the next and so F6P acts as substrate and a positive modulator. ATP, on the other hand, acts as a substrate and a negative modulator.

The various regulators of PFK act on these two curves in the following manner:



Negative modulators move the F6P curve to the right and decrease the enzyme-substrate affinity while positive ones make the curve more hyperbolic and so lower the K_m. Positive effectors tend to move the ATP curve up, and so reduce the effectiveness of inhibition while inhibitors drop it down and decrease the V_{max}. The 2 substrates interact on binding, ATP acting as a negative modulator for the F6P curve and F6P as a positive effector on the ATP situation. All other control features are superimposed on F6P or ATP regulation (42).

Control of PFK

PFK is acted on synergistically by a number of effectors and controlled by their combined effects. In the aerobic state, when fatty acids are the fuel, citrate and ATP levels are high and ADP, AMP, and P_i are low due to the high activity or oxidative phosphorylation. When O_2 is low, ATP levels drop as energy usage continues but production is curtailed and the products of ATP hydrolysis - ADP, P_i, and AMP - increase. This deinhibits PFK which is generally shut off. The rate in rabbit muscle increases 200-fold by mimicking

the metabolite changes occurring in vivo (51). Muscle PFK is more sensitive to these positive modulators than other PFKs from less glycolytic tissues. Functionally the most important contributor to the "flare up" of muscle glycolysis is product activation of PFK (52). Both FDP and ADP product activate the enzyme from muscle; since other tissues do not experience the large swings in activity common in muscle, it is not surprising that this regulatory property is generally absent in other tissue PFKs.

These control properties of muscle PFK are clearly the outcome of evolutionary action at this locus in metabolism. They are not typically seen, or are less exaggerated, in other tissues of the vertebrate body such as liver, red blood cells, or digestive tract (7). In most of these tissues, PFK catalytic activity is linear with time, whereas in muscle it follows an exponential product-activated pattern. In the case of most enzymes, including PFK from a protein-catabolizing slime mold (53), reaction products typically inhibit reaction velocity. Muscle PFK displays a 2- to 3-fold greater affinity for its substrate, F6P, than do PFKs from other tissues; under physiological conditions it will therefore compete for limiting substrate with a greater ability. Similar differences are noted in enzyme-substrate affinities for other glycolytic isozymes specific to muscle (25). Other distinguishing features of muscle PFK compared to tissues such as the liver include an overall "tighter" control by most organo-phosphate modulators and by citrate but a highly reduced sensitivity to ATP inhibition (54).

The autocatalytic activation of PFK in glycolysis is integrated with the next major control site in the pathway, pyruvate kinase, in that the product of the PFK reaction, ADP, is the substrate of the PK reaction. We will see

that in divers there is another important link operative here - FDP activation of PK.

Pyruvate Kinase

Pyruvate kinase isolated from different tissues exhibits kinetic properties of allosteric type. The isozyme L from liver (55) and the pyruvate kinase type II from kidney cortex (56) show a homotropic cooperative effect toward P-enolpyruvate and allosteric activation by fructose-1,6-P_2 and K^+; they are also allosterically inhibited by ATP and alanine. One of the isozymes from adipose tissue shows kinetic properties which resemble in some respects those of the L-type pyruvate kinase from liver (57). Finally, the pyruvate kinase type I from kidney cortex exhibits sigmoidal kinetics toward P-enolpyruvate but its activity is not affected by fructose-1,6-P_2 and ATP (56).

At present there is some kinetic evidence suggesting that the pyruvate kinase from muscle might have allosteric properties. This enzyme has been well characterized and its properties were extensively studied by Boyer (58). The protein is an oligomer formed by four subunits, which undergoes conformational changes upon a variety of experimental conditions.

Recently it was reported that pyruvate kinase from skeletal muscle and other M-type enzymes from different tissues are allosterically inhibited by phenylalanine and alanine (59). The in vivo control of pyruvate kinase activation of mammalian muscle is thought to be mediated by the storage form of energy, creatine phosphate. Creatine-P is not only a potent inhibitor of PK but also makes ATP a better inhibitor (61). Creatine-P has a K_i with respect to PEP of 2 mM and is non-competitive (60).

Turtles and Other Divers

Turtles are several times more tolerant of anoxia than other reptiles, which are in turn moere tolerant of anoxia than mammals (62) (Table 1). Several investigators have shown (63,64) that the red-eared turtle, Pseudemys scripta elegans, can live in a nitrogen atmosphere for up to a day with no ill effects. Although unable to extract oxygen from water, P. scripta survives dives of as long as two weeks at 16°-18°C despite total absence of electron transport chain mediated oxygen consumption. In Nature, Pseudemys is known to remain burrowed in the bottom mud of ice-covered lakes all winter in an anaerobic environment (65).

Jackson (66), on the basis of direct calorimetric measurements of metabolism, has divided the diving period of turtles into three metabolic phases (Figure 1):

(i) During phase I, lasting about a half hour at room temperature, the metabolic rate persists at the same rate as in the prediving condition, but O_2 tension rapidly falls.

(ii) During phase II, also lasting about 30 minutes, the metabolic rate falls to 40% of the initial rate and remaining O_2 reserves are exhausted.

(iii) The remainder of the dive (phase III) is totally anoxic. Jackson, on the basis of heat measurements, has estimated that this metabolism can yield 20% of the total energy available in the prediving state. In many diving vertebrates the length of the dive is largely determined by the rate of utilization of the internal O_2 store (10,12); but such is not the case in the diving turtle, P. scripta.

It has been shown that Pseudemys exhibits the diving syndrome: a) bradycardia commences soon after immersion, although it is often broken up by a

TABLE 1

Diving times for various species.

DIVERS

Species	Length of Dive
man	2-6 minutes
sea lion	5-6 minutes
porpoise	7-10 minutes
harbour seal	15-20 minutes
Weddell seal	60-77 minutes
sperm whale	60-90 minutes
bird/duck	1-3 minutes
reptiles/marine iguana	2 hours
turtle/*P. scripta*	days-months in winter

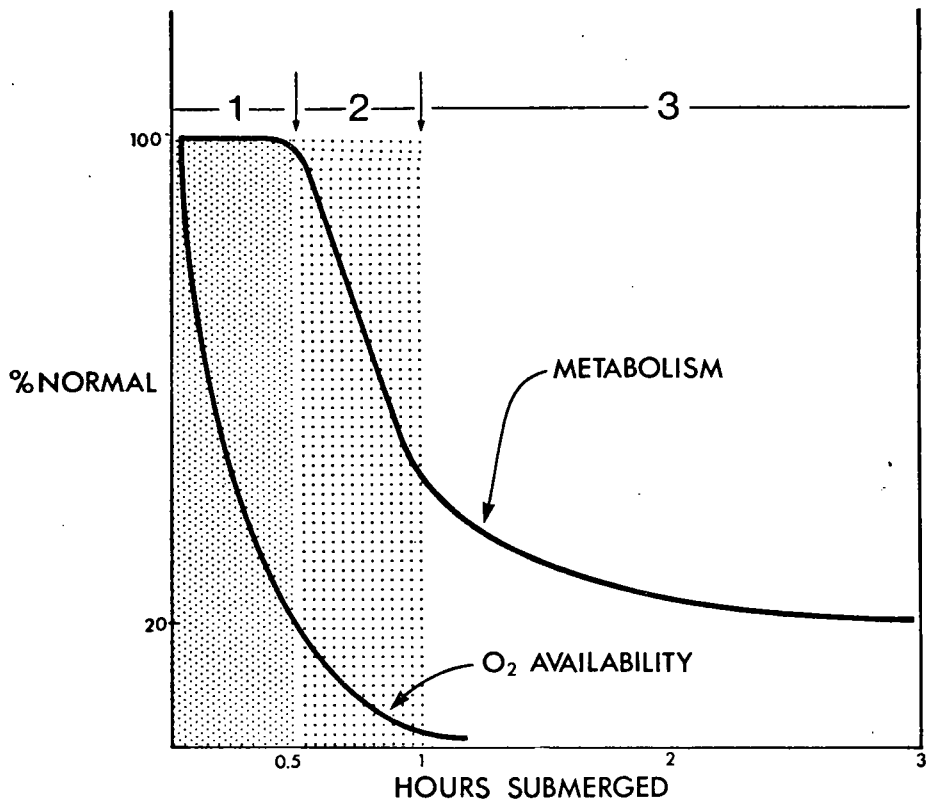


Figure 1. Decrease in O_2 availability and in metabolic rate of an aquatic turtle during a 3-hour dive at 24°C. Modified after (66).

series of rapid heart beats later in the dive (67); b) Weathers et al (68) have found that this turtle shows peripheral vasoconstriction that is used for thermoregulatory purposes as well as diving functions. Since P. scripta can survive long after all O_2 stores have been depleted the diving syndrome is probably most important to keep the brain, which is wholly dependent on blood glucose supply, well furnished with substrate while keeping the energy costs of the heart low.

Bing et al (69) have compared the intrinsic mechanical properties of isolated muscle preparations from turtle and rat hearts under identical experimental conditions and have shown that improved mechanical performance on the part of turtle heart muscle is primarily due to the increased glycolytic capacity of turtle myocardium. The possibility that energy costs of contraction in turtle hearts are less than for mammals was tested by blocking glycolysis in each of the hearts. If the differing tolerances to anoxia were primarily related to different energy requirements it might be expected that the decline in mechanical activity of turtle heart muscle during combined anoxia and glycolytic blockade would be considerably slower than that of the rat. Under these conditions however tension fell rapidly to zero, at least as rapidly in turtle preparations as in those from the rat.

Studied have shown that in Pseudemys heart the levels of the energy storage compound creatine phosphate began to decline during the first 3 hours of anaerobiosis whereas the levels of ATP stayed at their previous levels or actually rose (4, 35), even though in other vertebrate muscles a rapid decline in ATP concentrations is seen (33). Glycogen levels in the hearts of good vertebrate divers such as the seal are twice as high as the levels in non-divers' hearts (15,22). The turtle heart has 10 times as much glycogen

and these elevated levels are thought to help the turtle heart function in the initial stages of anoxia when blood glucose is still low (70).

The increased tolerance to hypoxia of turtle tissues largely reflects the enhanced capacity for anaerobic glycolysis present in the turtle ventricular myocardium. Belkin (5) showed the critical importance of anaerobic glycolysis to the anoxic turtle by poisoning glycolysis at triose-P dehydrogenase with iodoacetate and showing that turtles thus treated exhibited a greatly reduced tolerance to anoxia. The energetic inefficiency of glycolysis has been identified by several investigators as limiting in anaerobic survival where the heart and CNS must perform at zero or near zero oxygen tensions (66,4,69).

Reeves (71) perfused turtle hearts using uniformly labelled glucose C^{14} and found that added label was quantitatively (98%) converted to lactate, and neither dilution of exogenous glucose specific activity nor incorporation of label into glycogen or fatty acids occurs.

Other adaptations at the molecular level help explain the lack of vulnerability to hypoxia of the turtle heart: 1) The concentrations of 2 key control glycolytic enxymes, hexokinase and PFK, are nearly twice as high in the turtle heart as in other vertebrate hearts (71)(see Appendix). 2) Phosphorylase, the key enzyme in glycogen mobilization, is present in higher levels in its active form in turtle heart also (41). 3) The "heart" form of LDH is indistinguishable from the muscle type isozyme. It has taken on "muscle" M_4 kinetics as mentioned earlier and so is more "glycolytic" (72,73). Heart LDH is usually strongly substrate-inhibited so that whenever pyruvate concentrations rise, LDH is inhibited and pyruvate channelling into the Krebs cycle is in effect favored; this characteristic is missing in turtle

heart LDH, as in other diving species (73,74,75). Equally important, the LDH affinity for pyruvate decreases to the same range as in muscle. In other words, the kinetic properties of turtle heart LDH have been tailored to resemble the muscle isozyme, presumably because of the increased reliance of the heart upon anaerobic metabolism. 4) Turtles have also elaborated mechanisms to deal with the high levels of lactate accumulated. This high tolerance is due to large amounts of concentrated bicarbonate buffer, which allows for maintenance of intracellular pH in the turtle heart during the entire anoxic period (76). Blood pH drops by 1 unit whereas heart pH drops from 7.2 to a minimum after 12 hours of 6.8 (4).

Thus, in the turtle heart we have an organ with an extremely active anaerobic-glycolytic metabolism in contrast to the hearts of most vertebrates which display an almost total dependence on aerobic metabolism. Under 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 O_2 dependence, 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.

Since an impressive array of adaptations at the molecular level have already been seen in turtle heart, we looked at the glycolytic control points to see what takes place in "adaptive enzyme control".

CHAPTER II:

Materials and Methods

Materials

All substrates, cofactors, reagents, coupling enzymes, and resins were purchased from Sigma Chemical Co., St. Louis, Mo. Turtles used in this study were purchased from the NASCO Company Ltd., Fort Atkinson, Wisc., and were kept at 10°C in cold water. Ampholines, pH 3 to 10 and pH 5 to 8, were purchased from LKB Products (Stockholm, Brömvoa, Sweden). Starch for electrophoresis was purchased from Connaught Labs Ltd., Toronto, Canada.

Preparation and Assay of Turtle Heart PFK

Turtles were decapitated, their hearts excised, blotted, weighed, and cut up into homogenizing buffer: 1 mM Tris, 20 mM β-mercaptoethanol, 1 mM ATP, and 0.1 mM fructose-1,6-P_2. Hearts were homogenized in a Sorvall Omnimixer for 1 to 2 min. The homogenate was then centrifuged at 12,000 x g for 20 min and the pellet discarded.

The supernatant was adjusted to the correct ionic strength and pH and then put onto a DEAE-cellulose column equilibrated with 10 mM Tris, pH 8.0, 5 mM Mg^{2+}, 20 mM β-mercaptoethanol, 1 mM ATP, 0.1 mM fructose-1,6-P_2. A KCl gradient was run and the peak fraction collected. This partially purified phosphofructokinase was re-equilibrated and added to an hydroxylapatite column in 7 mM Tris, pH 7.6, 1 mM ATP, 0.1 mM fructose-1,6-P_2. Another KCl gradient (0-200 mM) was run and the peak fractions were collected in an LKB fraction collector. If enzyme free of substrate stabilizers was desired, ATP and fructose-1,6-P_2 were removed from the purification procedure. The final specific activity of the enzyme was 76 μmoles of fructose-1,6-P_2 produced per min per mg of protein at pH 7.55, about one-half that reported for crystalline beef heart phosphofructokinase (58). Our best preparations achieved about a 200-fold purification of the enzyme.

If purified in the presence of ATP and fructose-1,6-P_2 the final enzyme was stable for 10 to 14 days. However, in the absence of ATP half of the activity was lost in 1 day and in the absence of fructose-1,6-P_2 in about 1 hour. These preparations were free of enzymes which interfered with the measurement of either product of phosphofructokinase.

Dialysis of any preparation of purified enzyme resulted in complete loss of activity.

Phosphofructokinase was assayed via a coupled enzyme assay with dialyzed Sigma enzymes: either α-glycerophosphate dehydrogenase, triosephosphate, isomerase, and aldolase or pyruvate kinase and lactate dehydrogenase, and activity was measured by A_{340} decrease due to NADH oxidation. Imidazole buffers were used in all assay reactions. Standard assay mixtures contained the following in a final volume of 1 ml: 20 mM imidazole buffer, Mg^{2+}, K^{+}, ATP, fructose-6-P, NADH, and an excess of Sigma coupling enzymes. Saturating conditions for the reactions were: Mg^{2+}, 1 mM; K^{+}, 50 mM; ATP, 0.4 mM; fructose-6-P, 0.4 mM.

All reactions were started by the addition of the phosphofructokinase preparation. All experiments were performed at 25°C. All the spectrophotometric assays reported were repeated 2 to 3 times; change in O.D. was reproducible between 90-100% in all cases. K_m measurements of different metabolites for one enzyme prepraration if repeated were reproducible but varied between 3-5% when compared with different enzyme preparations.

Creatine Phosphate Control of Metabolism Studies

Crystalline rabbit muscle phosphofructokinase was obtained through Sigma Chemical Co., St. Louis. The phosphofructokinase was supplied as a suspension containing 1.3 M $(NH_4)_2SO_4$, 50 mM glycerophosphate, 4 mM

adenosine phosphate, and 1 mM dithiothreitol. The enzyme was diluted 40-fold prior to assay in a mixture of 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 50 mM β-mercaptoethanol and 2 mM Mg^{2+}. Under these conditions the enzyme is stable for several hours. In all experiments 5 µl of the diluted enzyme was used to start the reaction.

Standard assays for the determination of kinetic constants contained the following in a final volume of 1 ml: 50 mM Tris-HCl (pH 7.0) Mg^{2+}, K^{+}, fructose-6-phosphate, ATP, P-enolpyruvate, NADH, and excess dialyzed Sigma pyruvate kinase and lactate dehydrogenase.

Assays measuring the effects of simulated in vivo conditions contained in a final volume of 3 ml: 50 mM Tris-HCl (pH 7.2), various substrates and effectors as listed above, NADH and excess dialyzed Sigma alpha-glycerophosphate dehydrogenase, triosephosphate isomerase, and aldolase. All assays were performed at 25°C.

Preparation and Assay of Turtle Heart PK

Turtles were decapitated and hearts excised, blotted, weighed, and then cut up into ice-cold homogenization buffer.

Hearts were homogenized in a Sorvall Omnimixer for 1 to 2 min with 2 volumes of 0.01 M Tris-HCl buffer, pH 7.5, containing 20 mM β-mercaptoethanol, and 5 mM Mg^{2+}. The homogenate was then centrifuged at 12,900 x g for 20 min and the pellet discarded. The supernatant was then brought to 45% saturation with solid ammonium sulfate and stirred for 1 hour at 4°C. The suspension was then centrifuged as above, and the supernatant was brought to 48% saturation with solid ammonium sulfate. After 1 hour with stirring the solution was centrifuged at 35,000 x g for 20 min. The pellet was dissolved in a minimal volume of 1% glycine, pH 7.5, containing 20 mM β-mercaptoethanol.

After dialysis against 1% glycine, pH 7.5, the enzyme was electrofocused according to the method of Häglund (79). The enzyme was run at pH 5 to 8 (LKB-8133) at 700 volts for 18 to 24 hours. The temperature of the apparatus was maintained at 4 $\pm$0.05°. The purified enzyme had a specific activity of 210 µmoles of product formed per min per mg of protein at 25°C, compared to a specific activity of 250 to 280 µmoles of product formed per min per mg of protein at 30°C for crystalline pyruvate kinases from mammalian muscle (77). In early preparations a subsequent DEAE-cellulose step was performed. The enzyme was immobilized on the resin in 10 mM Tris-HCl buffer, pH 7.5, and then eluted with a KCl gradient (10-500 mM). This treatment did not, however, increase the specific activity relative to the peak tube of the electrofocused sample. The preparation was free of any enzymes that would either interfere with the basic assay or remove or interconvert any of the added metabolites.

Enzyme activity was assayed by the methods of Bücher and Pfleiderer (78). Pyruvate formation was coupled to lactate dehydrogenase and the rate of pyruvate kinase activity was measured as the decrease in A_{340} due to NADH oxidation. Imidazole buffers were used in all assay reactions. Standard assay mixtures contained the following in a final volume of 1 ml: 20 mM imidazole buffer, Mg^{2+}, K^{+}, ADP, P-enolpyruvate, NADH, and excess of Sigma lactate dehydrogenase at concentrations specified in the figure legends. Saturating concentrations for each of the reactants of the enzyme were 5 mM Mg^{2+}, 60 mM KCl, 0.7 mADP, 1.4 mM P-enolpyruvate. All reactions were performed at pH 6.80, 25°C and started by the addition of pyruvate kinase preparation.

Preparation and Assay of Turtle Heart Creatine Kinase (CK)

Hearts were homogenized with two volumes of 0.01 M Tris-HCl buffer, pH 7.5, containing 20 mM β-mercaptoethanol, and 5 mM Mg^{2+}. The homogenate

was then centrifuged at 12,900 x g for 20 minutes and the pellet discarded. The supernatant was then brought to 55% saturation with solid ammonium sulfate and stirred for 1 hour at 4°C. The suspension was then centrifuged as above, and the supernatant was brought to 65% saturation with solid ammonium sulfate. After one hour with stirring the solution was centrifuged at 35,000 x g for 20 min. The pellet was dissolved in a minimal volume of 1% glycine, pH 7.5, containing 20 mM β-mercaptoethanol. After dialysis against 1% glycine, pH 7.5, the enzyme was electrofocused according to the method of Häglund (79). The enzyme was run at pH 5 to 8 (LKB-8133) at 700 volts for 40 hours. The purified enzyme had a specific activity of 5 μmoles of product formed per min per mg protein at 25°C, compared to a specific activity of 53 μmoles of product formed per min per mg protein at 30°C for crystalline creatine kinases from mammalian muscle (80). The preparation representing about 23-fold purification was free of any enzymes that would either interfere with the basic assay or remove or interconvert any of the added metabolites. Adenylate kinase activity was zero under the assay conditions.

Horizontal starch electrophoresis was performed on 13% gels in Tris-Citrate buffer pH 7.0 by the method of Biewer (81). Gels were run at 250 volts and 10mamps for 18 hours at 4°C. Electrophoretic patterns were obtained with crude extracts and heart mitochondrial preparations (see Creatine Kinase chapter) of heart, skeletal muscle and brain from the adult turtle, Pseudemys scripta. The staining medium consisted of Tris buffer, 0.1 M, pH 7.5, 15 ml; $MgCl_2$, 0.1 M, 1.0 ml; glucose, 0.1 M, 1.0 ml; excess HK and G6PDH; creatine-P, 0.1 M, 0.6 ml; ADP, 0.05 M, 0.5 ml; NADP, 0.035 M, 0.5 ml; Nitro BT, 10 mg/ml, 1.0 ml; and PMS, 1 mg/ml, 0.12 ml.

In crude extracts of these tissues, adenylic kinase is also visualized by this method; it can be partially inhibited by the addition of AMP, 0.1 M, 1.0 ml, to the staining medium.

Preparation of Mitochondria

In the study of the cellular distribution of the enzymes, mitochondria were prepared by an adaptation of the method of Hogboom (82). Heart tissues were obtained and washed in a cold buffer medium pH 7.2 at 4°C containing 0.2 M Tris-HCl, 1 mM $MgCl_2$, 0.025 M sucrose. All subsequent operations were conducted at 0° to 4°C. After grinding in the Sorvall Omnimixer for 1 to 1.5 min, the tissue was filtered through cheese cloth. The crude homogenate was centrifuged in a Sorvall superspeed RC 2-B centrifuge for 10 min at 650 x g. Following centrifugation, the tissue pulp was discarded and the supernatant was centrifuged at 7,000 x g. The pellet from the preceding step was resuspended and washed in the homogenizing medium. After several cycles of washing and centrifuging, the mitochondrial pellet was obtained by centrifugation in the homogenizing medium at 24,000 x g. The suspension of mitochondria so obtained was frozen and thawed three times, then centrifuged and the supernatant was used as the source of the mitochondrial enzyme.

Enzyme activity was measured by the coupled assay technique. For the "forward" direction, ATP production was coupled to HK and G6PDH and the rate of creatine kinase activity was measured as the increase in A_{340} due to NADP reduction. For the "reverse" direction ADP production was coupled to pyruvate kinase and lactate dehydrogenase and the rate of oxidation of NADH was measured. Imidazole buffers were used in all assay reactions. Standard assay mixtures contained the following in a final volume of one ml: 20 mM

imidazole buffer, Mg^{2+} (the diacetate salt of magnesium was used in all experiments as it has been shown (83) that other common anions such as chloride and sulfate inhibit the enzyme), creatine-P, ADP, NADP, HK, G6PDH at pH 6.7 for the forward reaction. For the reverse direction in one ml; 20 mM glycine-NaOH buffer pH 9.0, Mg^{2+}, creatine, ATP, P-enolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase. All reactions were performed at 25°C and started by the addition of the creatine kinase preparation. Saturating concentrations for each of the reactants of the cytosol enzyme are: 5 mM Mg^{2+}, 5 mM creatine-P, 0.8 mM ADP, 4 mM glucose. Saturating concentrations for the mitochondrial enzyme are: 5 mM Mg^{2+}, 5 mM creatine-P, 0.2 mM ADP, 4 mM glucose.

Protein Determinations

Protein concentrations were determined spectrophotometrically by the method of Waddell (84). Readings at 215 nm and 225 nm were taken for each sample and determined by the formula (215-225) x 0.144 = mg/ml. Initially these measurements were checked against the method of Lowry (85) and another spectrophotometric determination (86). Results of all three methods were the same within experimental error.

CHAPTER III:

Phosphofructokinase

In many diving vertebrates the length of the dive is largely determined by the rate of utilization of this internal oxygen store (10,12); but such is not the case in the diving turtle, Pseudemys scripta.

Operationally this species can be classified as a facultative anaerobe since it utilizes oxygen when available yet can dive anaerobically for as long as two-week periods despite the total absence of electron-transport mediated oxygen consumption (87, 7). Although it undergoes the typical diving syndrome during first phases of prolonged diving, later stages are totally anoxic (66). Blood sugar levels rise during this prolonged anoxia from 3 mM to 58 mM, blood lactate rises from 6 mM to nearly 125 mM, and blood pH drops from pH 7.9 to pH 6.8 (88). Unlike the typical vertebrate heart, the anoxic turtle heart can achieve steady state work rates fully equivalent to the aerobic performance. Under these conditions, current evidence indicates that most energy demands of the anoxic heart are met by glycolytic mechanisms (40). Thus, in the turtle heart we have an organ with an extremely active anaerobic metabolism, in contrast to the heart of most vertebrates, which displays an almost total dependence upon aerobic metabolism. By comparing this system to the typical mammalian heart, we felt that we may be able to gain some useful insights into selective forces involved in the evolutionary development of an extreme tolerance to anoxia.

In this context, we turned our attention to the catalytic and regulatory properties of turtle heart phosphofructokinase, a key glycolytic enzyme

involved in the control of glycolysis in many tissues (89,90). We found that the enzyme is clearly of the allosteric type, displaying a number of general control features that are similar to the mammalian homolog. However, the turtle enzyme is largely insensitive to inhibitory control by high ATP concentrations, unlike heart and muscle phosphofructokinases of mammals. The apparent K_i for turtle heart phosphofructokinase is about 40-fold greater than the mammalian heart phosphofructokinase (91), and about 3-fold greater than for the mammalian muscle enzyme (91). The usual ATP inhibitory control appears to be taken over by creatine phosphate, which is a potent inhibitor of the enzyme at all pH values studied. Equally significant, the creatine phosphate inhibition is strongly reversed by fructose-1,6-P_2. Together, these two effects seem to supply a very sensitive potential control mechanism for phosphofructokinase function in a glycolytic tissue which displays little if any change in ATP concentration during aerobic-anaerobic transition (35).

RESULTS

Specific Activity

Turtle hearts were found to contain an average of 18.9 units of phosphofructokinase per gram wet weight of tissue compared to 110 units for rat muscle (91), 22 units for sheep heart (77), and one unit for rat liver (91).

pH Effects

Turtle heart phosphofructokinase has a pH optimum of 7.55 in either 50 mM imidazole, 50 mM Tris or 20 mM triethanolamine buffers, whereas the optimal pH for mammalian muscle phosphofructokinase is pH 8.2 (42).

TABLE I

Effect of various nucleoside triphosphates on turtle heart phosphofructokinase

Reaction concentrations are: 1.5 mM Mg^{2+}, 50 mM KCl, 1 mM fructose-6-P, 0.15 mM NADH, excess of coupling enzymes, and increasing concentrations of the nucleoside triphosphate listed.

Nucleoside triphosphate	V_{max}	K_m
	%	mM
ATP	100	0.026
GTP	64	0.123
ITP	83	0.123

Turtle heart enzyme also shows a much broader pH optimum and exhibits at least 60% of maximal activity at pH values as low as 6.80. To facilitate comparison of the turtle and mammalian enzymes, we studied turtle heart phosphofructokinase at pH 7.90 as well as at pH 6.8, the intracellular pH of anoxic turtle heart (4).

Enzyme at pH 7.90 (Purified in presence of both ATP and fructose-1,6-diP)

At pH 7.90, turtle heart phosphofructokinase exhibits Michaelis-Menten

kinetics with respect to Mg^{+2}, Mn^{+2}, fructose-6-P, ATP, and the other nucleoside triphosphates. All Michaelis constants (K_m values) were determined from Lineweaver-Burk plots and the values are 0.175 mM, 0.040 mM and 0.026 mM for Mg^{+2}, fructose-6-P F6P and ATP respectively. The corresponding values from sheep heart phosphofructokinase (44) are 0.170 mM, 0.035 mM and 0.014 mM. Very little ATP inhibition was observed at pH 7.90. At an ATP concentration of 8 mM some inhibition of the enzyme was observed. The activity of the enzyme in the presence of different nucleosides was tested. Table 1 shows that GTP or ITP could serve as phosphate donor almost as well as ATP. The affinity of the enzyme for both of these substrates was slightly lower as was the maximum velocity (V_{max}) obtained.

The affinity of the enzyme for fructose-6-P was lowered by using ITP (K_m for fructose-6-P being 0.063 mM) or GTP (K_m for fructose-6-P being 0.080 mM) instead of ATP, but upon the addition of 0.05 mM ADP, a product of the normal phosphofructokinase reaction, the K_m for fructose-6-phosphate was decreased to the normal value, 0.030 mM, with either triphosphate.

Various potential modulators (e.g. 0.5 mM P-enolpyruvate, AMP, 3P-glyceric acid, cyclic AMP) did not affect the K_m or V_{max} for any of the substrates; however, addition of 0.5 mM citrate increased K_m of fructose-6-P almost 2.5 fold, to 0.105 mM.

The Michaelis constant for Mn^{+2} is 0.026 mM which is significantly less than that for Mg^{+2} but the V_{max} is 50% of that with magnesium. In the presence of manganese the K_m for fructose-6-P is 0.030 mM.

General Properties at pH 6.8

After 12 hours of exposure to pure nitrogen the intracellular pH of P. scripta heart drops to about 6.80 (4); therefore this pH was chosen to study the catalytic and regulatory parameters of the enzyme more fully.

Table 2 shows that at pH 6.8, compared to pH 7.9, the K_m for ATP is slightly lower, the K_m for Mg^{++} lower by an order of magnitude, and the K_m for fructose-6-P increased from about 0.04 to 0.06 mM. With Mn^{++} as the cofactor, the K_m for ATP is reduced again by a factor of about 3, while the K_m for fructose-6-P is about halved. However, in the presence of Mn^{++}, the maximum velocity is only about 75% of that in the presence of Mg^{++}. In contrast

TABLE II

Michaelis constants for substrates of phosphofructokinase reaction

Constant in every determination were 50 mM K^+ and 0.15 mM NADH. The optimal concentrations of each substrate not varied were 1.5 mM Mg^{2+}, 0.4 mM ATP, 1 mM fructose-6-P, 0.2 mM Mn^{2+}. K_m and V_{max} values were obtained from Lineweaver-Burk plots.

Substrate or cosubstrate	K_m	V_{max}
	mM	%
MgATP	0.019	100
Fructose-6-P (with Mg^{2+})	0.060	100
Mg^{2+}	0.018	100
Mn^{2+}	0.011	75
MnATP	0.006	75
Fructose-6-P (with Mn^{2+})	0.034	75

to the sheep heart enzyme, which shows sigmoidal fructose-6-P saturation kinetics in this pH range (42), the turtle heart enzyme displays hyperbolic saturation kinetics with respect to both substrates. As in the case of

the enzyme at pH 7.9, at pH 6.8 all 3 nucleosides tested seemed to be about equally effective as phosphate donor for the transferase reaction.

Citrate Inhibition at pH 6.8

Like the mammalian homolog (42), turtle heart phosphofructokinase is potently inhibited by citrate. Phosphofructokinase inhibited by 0.5 mM citrate shows sigmoidal kinetics with an $[S]_{0.5}$ of 0.07 mM for fructose-6-P and an n value of 2.4. On the addition of 0.1 mM cyclic AMP, 0.25 mM AMP, or 1 mM ADP, hyperbolic saturation curves return with K_m values of 0.038 mM, 0.070 mM and 0.070 mM respectively (Figure 1).

ATP inhibition at pH 6.8

ATP inhibition, as expected, was greater at pH 6.80 than at 7.90. The concentration of ATP necessary to reduce rabbit muscle phosphofructokinase activity to one-half of V_{max} was found to be 3.1 mM (91). For turtle heart enzyme that concentration is 8.5 mM showing this enzyme to be less effectively inhibited by ATP. Figure 2 shows that ATP inhibition is not lessened at increasing concentrations of fructose-6-P but is almost totally reversed by the addition of 0.1 mM fructose-1,6-diP even at low fructose-6-P concentrations.

Concentrations of 2 mM ATP inhibit the enzyme, causing the fructose-6-P saturation curve to become sigmoidal with an $[S]_{0.5}$ of 0.095 mM and an n value of 1.8, but do not affect the V_{max}. Upon addition of 0.25 mM AMP or 0.1 mM cyclic AMP (Figure 3) hyperbolic curves were obtained with a K_m of 0.065 mM for fructose-6-P. Addition of 0.1 mM ADP (Figure 4) to the ATP inhibited enzyme also gave a hyperbolic curve with a K_m of 0.083 mM for fructose-6-P.

Figure 1. Effect of cAMP, AMP, and ADP in reversing citrate inhibition of turtle heart phosphofructokinase at pH 6.8. Reaction conditions are: 1.5 mM Mg^{2+}, 50 mM K^{+}, 0.4 mM ATP, increasing fructose-6-P (F6P), 0.15 mM NADH, and excess coupling enzymes. Additions are: ▲ , none; ●, 0.5 mM citrate; ○, 0.5 mM citrate plus 0.25 mM AMP or 0.1 mM ADP; Δ , 0.5 mM citrate plus 0.1 mM cAMP.

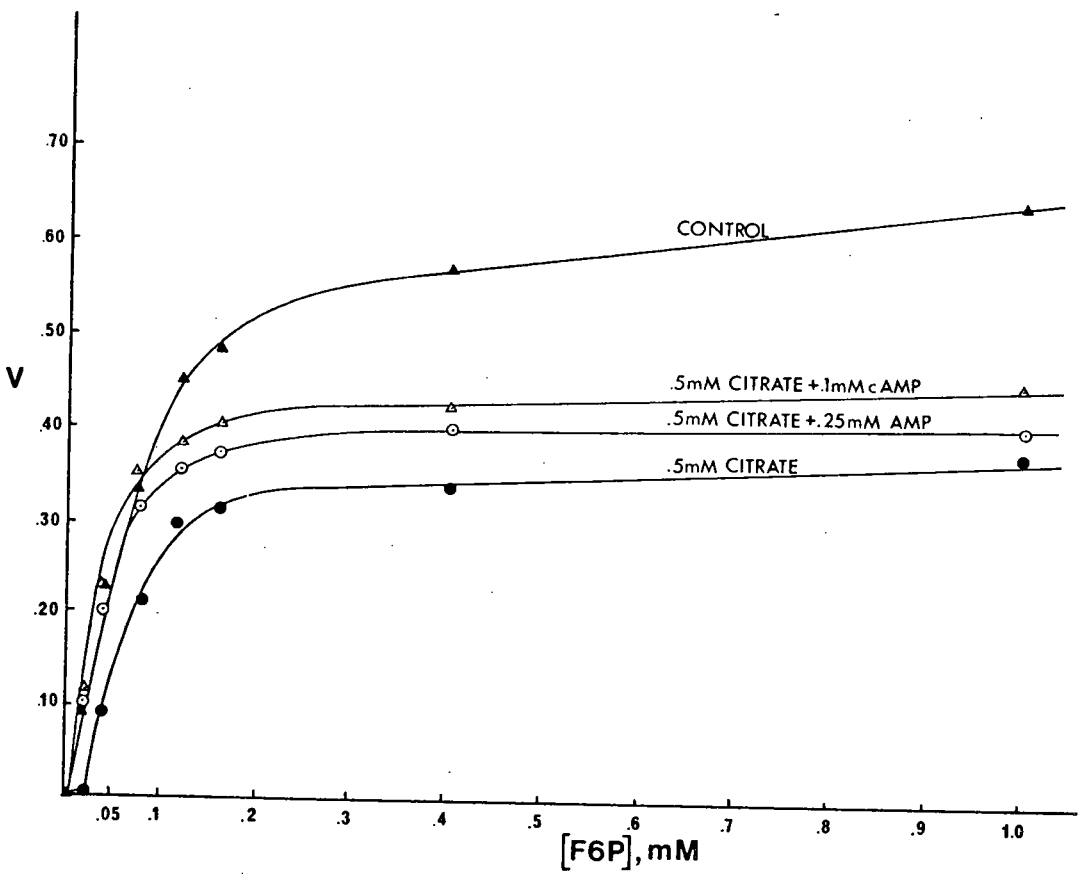

.70
.60
.50
.40
.30
.20
.10
V
CONTROL
.5mM CITRATE +.1mM cAMP
.5mM CITRATE +.25mM AMP
.5mM CITRATE
.05
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
[F6P], mM

Figure 2. Effect of fructose-6-P and fructose-1,6-P_2 at pH 6.8 on ATP inhibition of turtle heart enzyme purified in the absence of fructose-1,6-P_2. Reaction concentrations are: 1.5 mM Mg^{2+}, 50 mM K^+, 0.4 mM ATP, 0.15 mM NADH, excess of coupling enzymes, and fructose-6-P at concentrations below: ●, 0.4 mM fructose-6-P; ○, 0.2 mM fructose-6-P; □ , 0.1 mM fructose-6-P; △ , 0.04 mM fructose-6-P; ▲ , 0.04 mM fructose-6-P plus 0.1 mM fructose-1,6-P_2. Reaction velocity (V) is expressed in terms of ΔA_{340} per min.

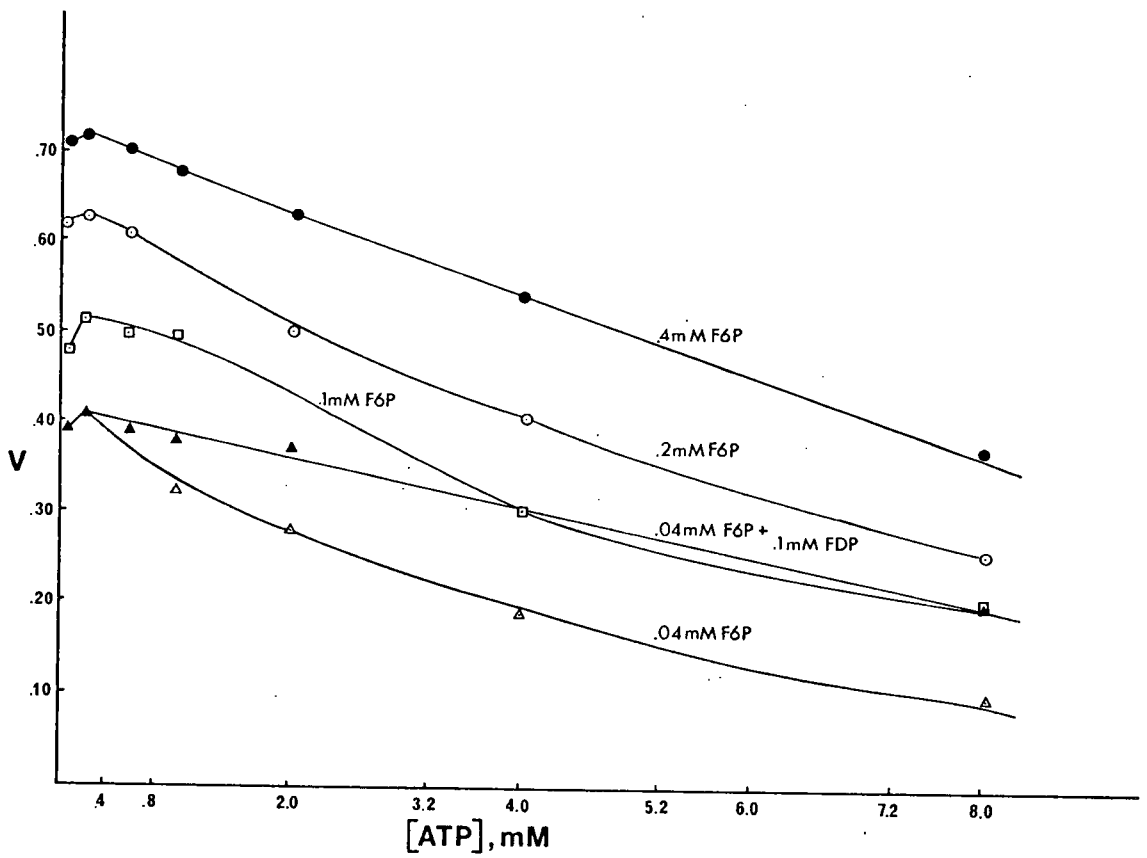

.70
.60
.50
.40
.30
.20
.10
V
.4mM F6P
.2mM F6P
.1mM F6P
.04mM F6P + .1mM FDP
.04mM F6P
.4
.8
2.0
3.2
4.0
5.2
6.0
7.2
8.0
[ATP], mM

Figure 3. Effect of ATP, AMP, and cAMP on fructose-6-P (F6P) saturation kinetics. Reactant concentrations are: 1.5 mM Mg^{2+}, 50 mM K^{+}, 0.4 mM ATP, variable fructose-6-P, 0.15 mM NADH, excess coupling enzymes. Conditions are: ▲ , optimal ATP; 0, 2 mM ATP; Δ , 2 mM ATP plus 0.25 mM AMP; ⊡ , 2 mM ATP plus 0.2 mM cAMP. Turtle heart phosphofructokinase purified in presence of stabilizers and assayed at pH 6.8.

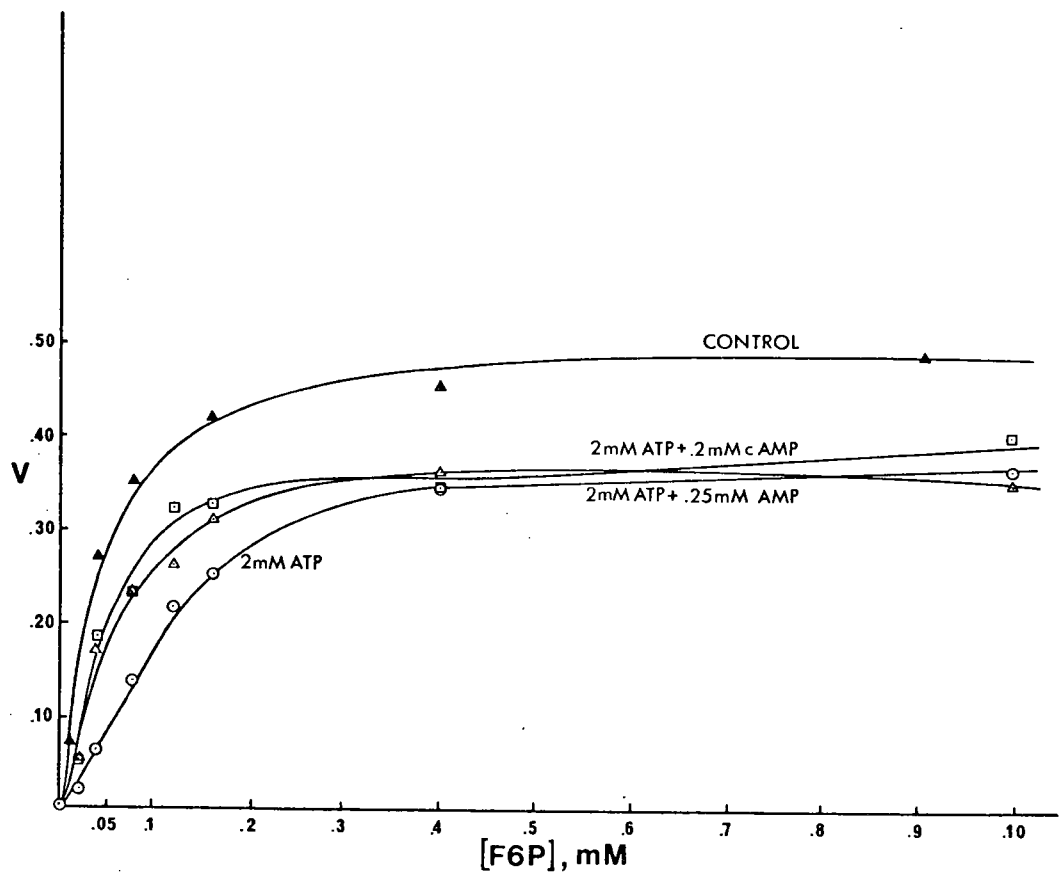
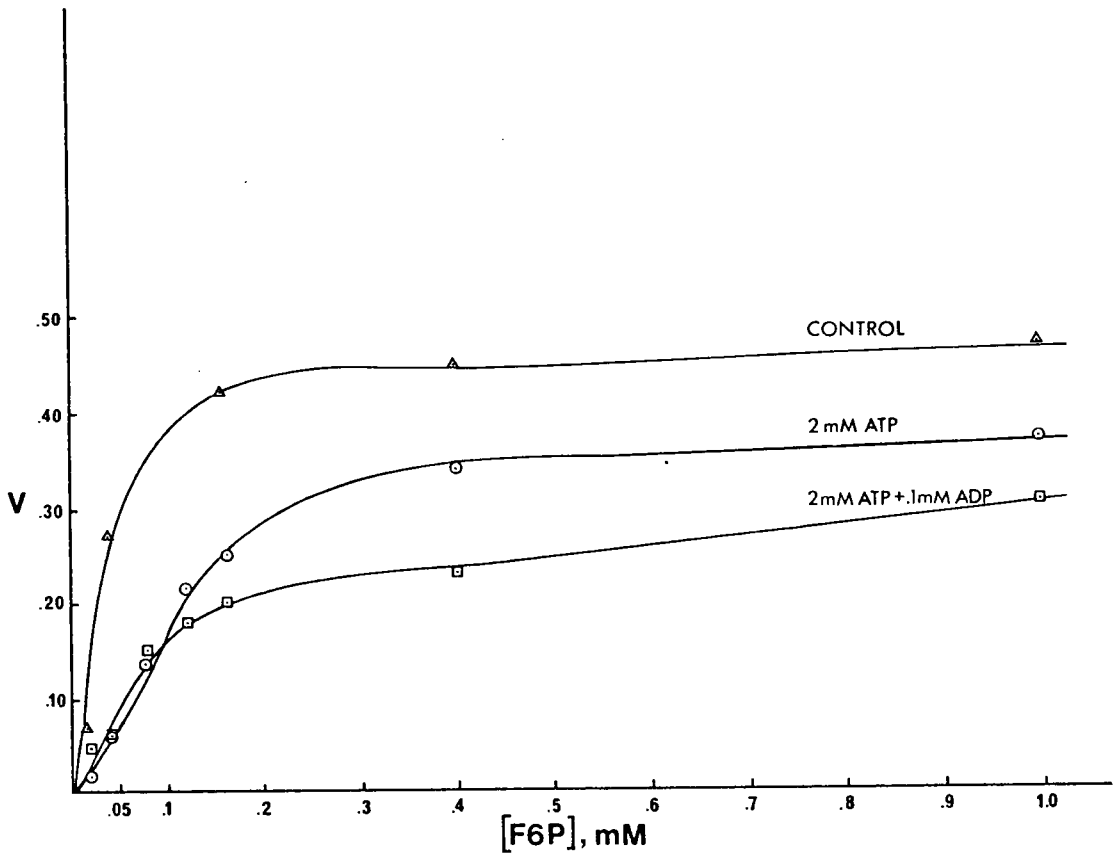

CONTROL
2mM ATP + .2mM c AMP
2mM ATP + .25mM AMP
2mM ATP
V
.50
.40
.30
.20
.10
.05
.1
.2
.3
.4
.5
.6
.7
.8
.9
.10
[F6P], mM

Figure 4. Effect of ADP in reversing ATP inhibition of turtle enzyme at pH 6.80. Reaction conditions are: 1.5 mM Mg^{2+}, 50 mM K^{+}. ATP as described, varying fructose-6-P (F6P) concentrations, 0.15 mM NADH, excess coupling enzymes. Additions are: Δ , optimal (0.4 mM) ATP; 0, 2 mM ATP; ⊡ , 2 mM ATP plus 0.1 mM ADP.


CONTROL
2 mM ATP
2mM ATP +.1mM ADP
V
.50
.40
.30
.20
.10
.05
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
[F6P], mM

The enzyme purified in the absence of stabilizers, ATP and fructose-1,6-diP, shows identical K_m values for all substrates and the same pH optimum, but differs in ATP inhibition characteristics in much the same way as Mansour and Ahlfors (44) have reported for mammalian heart phosphofructokinase in that removal of most ligands bound to the purified enzyme results in increased sensitivity of the enzyme to ATP inhibition.

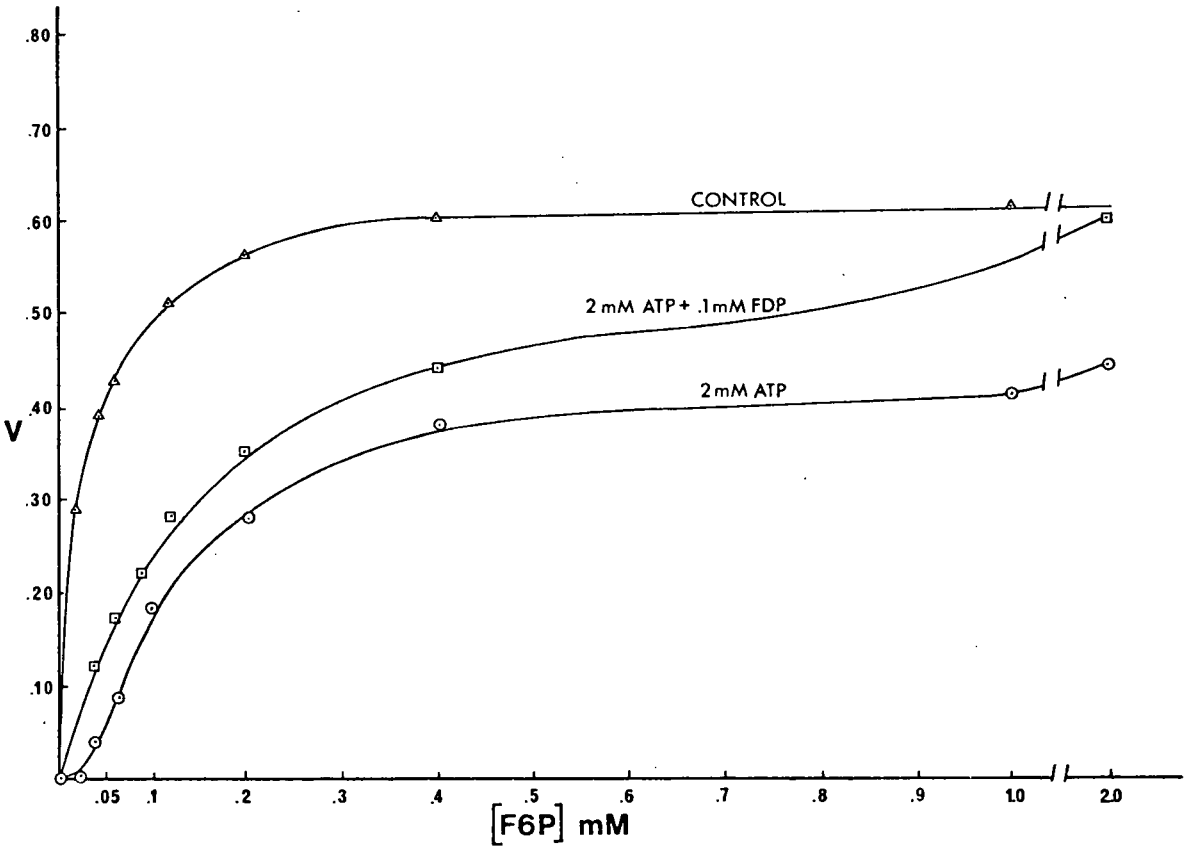
For the enzyme purified without fructose-1,6-diP or ATP the concentration of ATP required to reduce the reaction velocity to one-half V_{max} is 6.3 mM.

To test the effects of fructose-1,6-diP on ATP inhibition, phosphofructokinase was purified in the absence of fructose-1,6-diP and assayed by coupling to Sigma pyruvate kinase and lactate dehydrogenase. The inhibited enzyme (in the presence of 2 mM ATP) showed sigmoidal kinetics with an $[S]_{0.5}$ of 0.144 for fructose-6-P, and $\underline{n}$ value of 1.8, and 77% of V_{max} activity. Upon addition of fructose-1,6-diP the curve became hyperbolic again with a K_m value of 0.145 mM and restoration of 100% of V_{max} (Figure 5).

Search for Other Potential Modulators

Because of the low sensitivity of the turtle heart phosphofructokinase to ATP (apparent K_i of 8.3 mM compared to a value of 0.1 mM for the sheep heart homolog), a brief analysis of the effects of a variety of other compounds was made. Of the series tested, fructose-1-P, glucose-6-P, α-glycerophosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, several amino acids, other nucleoside triphosphates, and creatine phosphate, the latter showed by far the most potent effect on turtle heart phosphofructokinase.

Figure 5. Effect of fructose-1,6-P_2 on ATP inhibition of turtle heart phosphofructokinase purified in the absence of fructose-1,6-P_2. Reactant concentrations are the same as described for Figure 3. Additions are: Δ , none; 0, plus 2 mM ATP; □ , 2 mM ATP plus 0.1 mM fructose-1,6-P_2.


.80
.70
.60
.50
.40
.30
.20
.10
V
CONTROL
2 mM ATP + .1mM FDP
2mM ATP
.05
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
2.0
[F6P] mM

Creatine Phosphate Inhibition at pH 6.8

Figure 6 shows that at 2 mM creatine-P, the velocity of the reaction is reduced to one-half of V_{max} and by 7 mM only 25 percent of V_{max} activity remains. The addition of 0.1 mM fructose-1,6-diP significantly reverses the effect so that 12 mM creatine-P is necessary to reduce the reaction to one-half V_{max}. Fructose-6-P saturation curves are profoundly affected at even 1 mM creatine-P (Figure 7). The saturation curve becomes very sigmoidal with a $[S]_{0.5}$ value of 0.23 mM and there is a large reduction on V_{max}. The ability of various compounds to reverse the inhibition can be seen in Figures 8 and 9, and are summarized in Table 3. Of these, fructose-1,6-diP and cyclic AMP are the most potent; both can completely reverse the inhibition, and both induce the enzyme to display hyperbolic saturation kinetics. To facilitate comparison between the turtle heart enzyme and other phosphofructokinases a chart is provided (page 64).

DISCUSSION

To date, although the extreme anoxia tolerance of diving turtles such as P. scripta is widely recognized (62,73), there have been few attempts to work out underlying enzymatic mechanisms. However, the following facts are apparently well established: (a) during anoxia, the heart relies largely upon glycogen as the storage form of energy, (b) the utilization of labelled glucose is quantitatively accounted for by the accumulation of labelled lactate(40), (c) during aerobic-anaerobic transition in perfused turtle heart, ATP concentrations do not show even a transient change (35) unlike the situation in perfused rat heart (92), and (d) creatine phosphate concentrations, in contrast, drop dramatically during aerobic-anaerobic transition (4). In this

Figure 6. Effect of fructose-1,6-P_2 in reversing creatine-P inhibition at pH 6.8 of turtle phosphofructokinase purified without fructose-1,6-P_2. Reactant concentrations are: 20 mM imidazole buffer, 1.5 mM Mg^{2+}, 50 mM K^{+}, 0.4 mM ATP, 1 mM fructose-6-P, 0.15 mM NADH, increasing concentrations of creatine-P, and excess coupling enzymes. 0, no additions; Δ , plus 0.1 mM fructose-1,6-P_2.

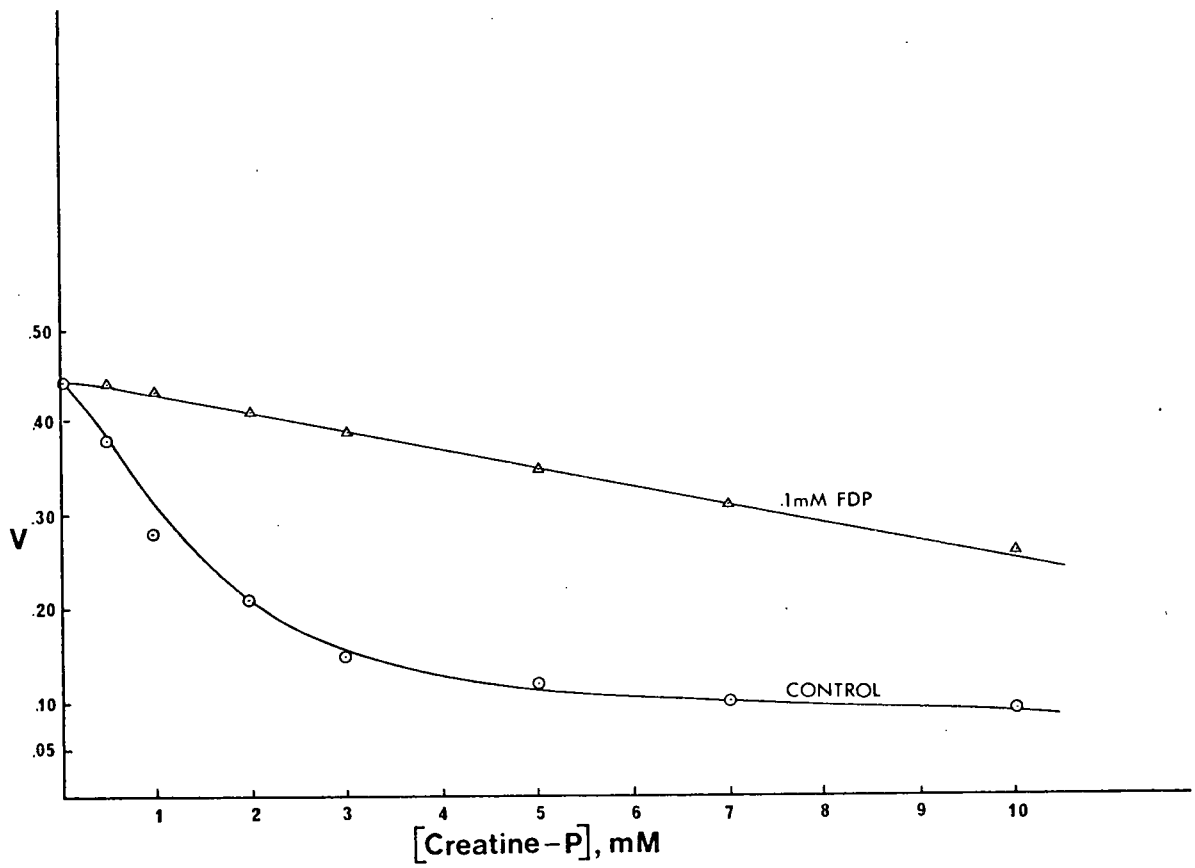

.50
.40
.30
.20
.10
.05
V
.1mM FDP
CONTROL
1
2
3
4
5
6
7
8
9
10
[Creatine-P], mM

Figure 7. Effect of fructose-1,6-P_2 and creatine-P on fructose-6-P (F6P) saturation kinetics for turtle heart phosphofructokinase at pH 6.80 purified in the absence of fructose-1,6-P_2. Reaction concentrations are the same as in Figure 5. Additions are: ⊡ , none; ○, 1 mM creatine-P; △ , 1 mM creatine-P plus 0.1 mM fructose-1,6-P_2.

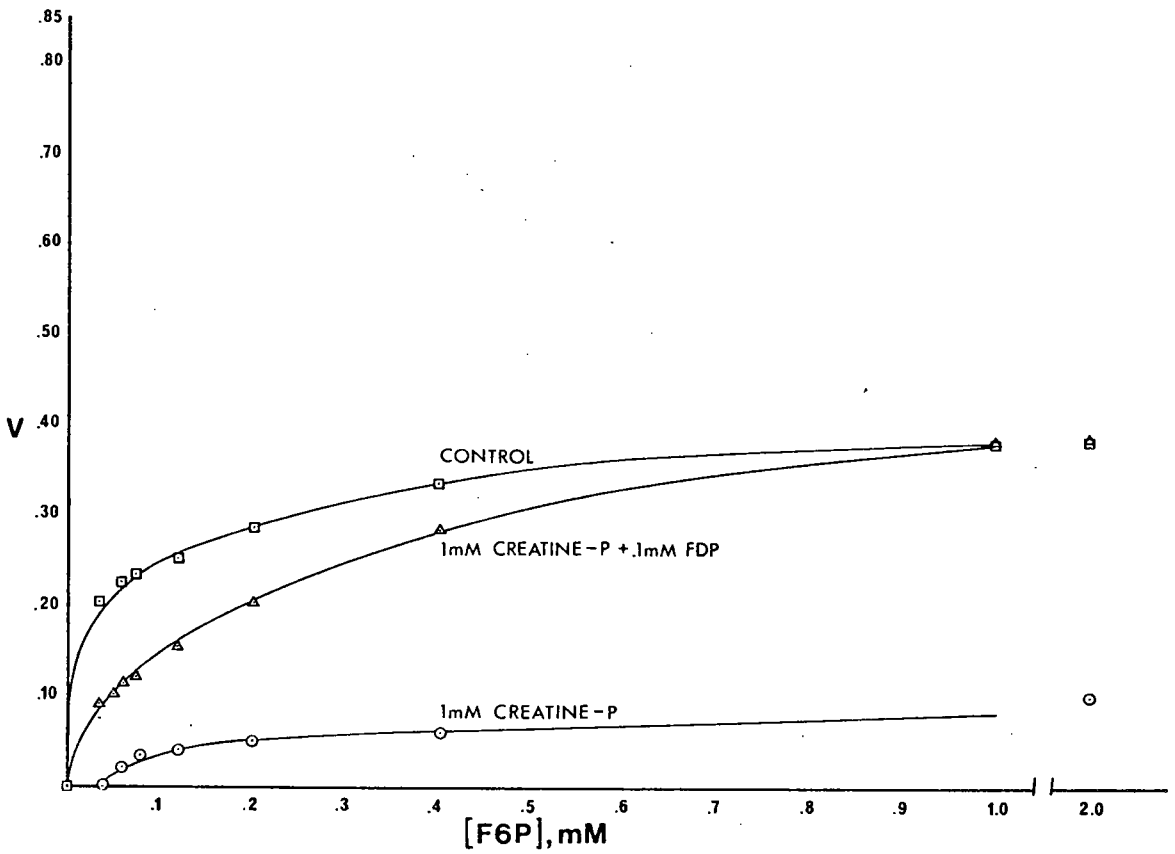

.85
.80
.70
.60
.50
V .40
.30
.20
.10
CONTROL
1mM CREATINE-P +.1mM FDP
1mM CREATINE-P
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
2.0
[F6P], mM

Figure 8. Effect of cAMP, AMP, and 3-phosphoglyceric acid in reversing creatine-P inhibition of phosphofructokinase at pH 6.8. Reaction conditions are as in Figure 5. Additions are: ●, none; ○, 1 mM creatine-P; □, 1 mM creatine-P plus 0.5 mM AMP; △, 1 mM creatine-P plus 1 mM 3-phosphoglycerate; ▲, 1 mM creatine-P plus 0.1 mM cAMP. F6P, fructose-6-P.

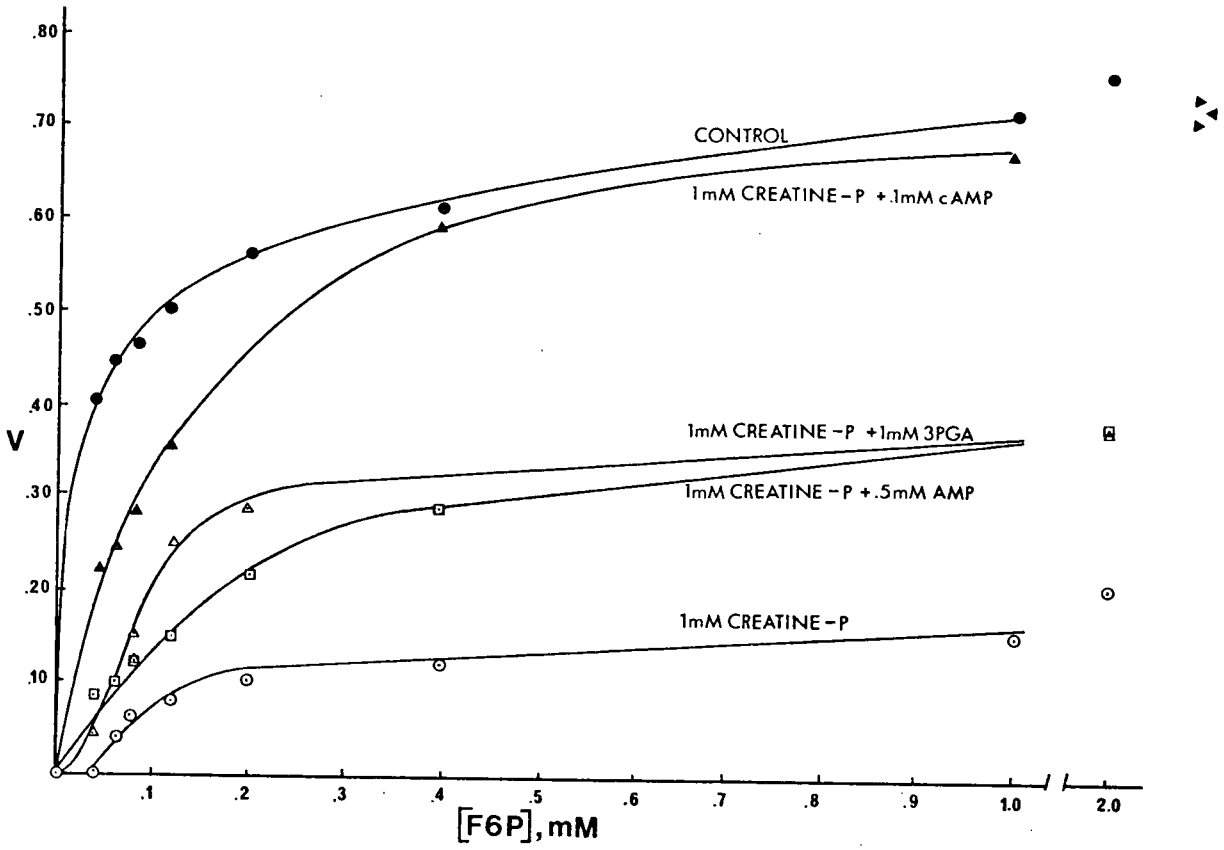
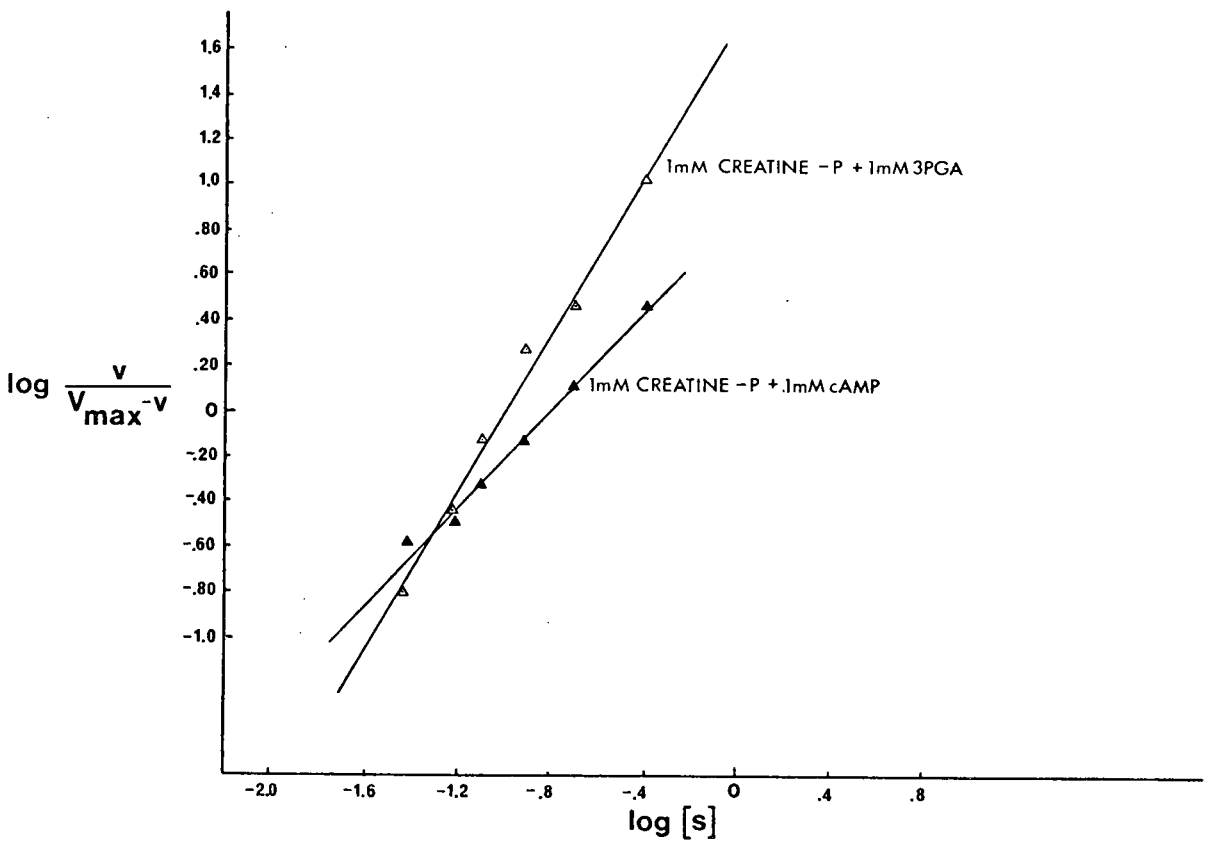

.80
.70
.60
.50
.40
.30
.20
.10
V
CONTROL
1mM CREATINE-P +.1mM cAMP
1mM CREATINE-P +1mM 3PGA
1mM CREATINE-P +.5mM AMP
1mM CREATINE-P
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
2.0
[F6P], mM

Figure 9. Hill plots of AMP and 3-phosphoglyceric acid reversal of creatine-P inhibition of enzyme at pH 6.8. Again Δ, 1 mM creatine-P plus 1 mM 3-phosphoglycerate; ▲, 1 mM creatine-P plus 0.1 mM cAMP. Reaction conditions and additions are the same as for Figure 8.


log v / (Vmax - v)
1.6
1.4
1.2
1.0
.80
.60
.40
.20
0
-.20
-.40
-.60
-.80
-1.0
1mM CREATINE - P + 1mM 3PGA
1mM CREATINE - P + .1mM cAMP
-2.0
-1.6
-1.2
-.8
-.4
0
.4
.8
log [s]

biological context, the information which we have obtained on turtle heart phosphofructokinase can be classified into two broad categories.

One set of special or selected properties of the enzyme appear to be quite unique to this organism and seem to fit the enzyme for function in its particular cellular environment. Included in this set of properties are the enzyme's sensitivity to ATP and to creatine phosphate. Compared to other phosphofructokinases, the turtle heart enzyme appears to be surprisingly insensitive to ATP. It is about 40 fold less sensitive than sheep heart phosphofructokinase when the latter is purified in the presence of metabolite stabilizers, and about 200 fold less sensitive than sheep heart phosphofructokinase when the latter is purified in the absence of the same stabilizers (44). Turtle phosphofructokinase is about 1/3 as sensitive to ATP as is the rat muscle enzyme (91), and about 1/4 as sensitive to ATP as is rat liver phosphofructokinase (91). Thus there appears to be a gradation of phosphofructokinase sensitivity to ATP depending upon the glycolytic capacity (or dependence) of the tissue, with turtle heart appearing at one end of the spectrum. Since ATP concentrations do not change during aerobic-anaerobic transition in turtle heart (35), ATP is not a useful metabolite signal of the cell's requirements for glycolytic activation; hence in this situation, the extreme ATP insensitivity of the enzyme makes biological sense and is probably the outcome of evolutionary selection acting on this locus in metabolism.

In contrast, creatine phosphate concentrations could serve as a useful indication of the cell's requirements for glycolysis, since creatine phosphate levels, quite high in the aerobic turtle heart, drop off

dramatically at the onset of hypoxic work (4). According to our data on phosphofructokinase, this drop in creatine phosphate level in itself would serve to deinhibit the enzyme, and this deinhibition is strongly potentiated by fructose-1,6-diP concentrations very much within the biological range (35). As fructose-1,6-diP is a product of the phosphofructokinase reaction, it is evident that taken together, creatine phosphate and fructose-1,6-diP control of this enzyme raise the potential for a highly efficient, autocatalytic increase in the rate of phosphofructokinase activity in this tissue during aerobic-anaerobic transition. In effect, the role of ATP inhibition and its reversal by effectors, which is so important to mammalian muscle and heart phosphofructokinases seems to have been taken over in the turtle heart by creatine phosphate. In addition to fructose-1,6-diP, a number of other metabolites can effectively contribute to reversing creatine phosphate inhibition. Of these, cyclic AMP is probably the least significant, since our concentrations are substantially higher than known in vivo levels (93). However, AMP and 3-phosphoglycerate may contribute to the in vivo reversal of creatine phosphate inhibition.

Parenthetically, it should be mentioned that the degree to which phosphofructokinases depend upon creatine phosphate levels also appears to correlate with the anoxia tolerance of the tissue. Thus, in contrast to the turtle enzyme, the rat heart enzyme is largely refractory to creatine phosphate (P. J. Randle, personal communication), while rabbit muscle phosphofructokinase is 20% inhibited by 1 mM creatine phosphate, and 44% at about 3 mM creatine phosphate (94).

In many of its other kinetic characteristics, turtle heart phosphofructokinase appears to be similar to the enzyme from other sources. Included in

TABLE III

Effect of various metabolites on turtle heart enzyme inhibited with 1 mM creatine-P

Reaction concentrations are 1.5 mM Mg^{2+}, 50 mM K^{+}, 0.4 mM ATP, 0.15 mM NADH, increasing fructose-6-P concentrations, excess coupling enzymes, and additions as indicated in the table.

Compound added to creatine-P-inhibited enzyme	Concentration	K_m (fructose-6-P) or $[S]_{0.5}$	n	V_{max}	Kinetics
	mM			%	
None		0.23		25	Sigmoidal
AMP	0.5	0.10	1.1	55	Sigmoidal
3PGA[a]	1	0.158	1.72	55	Sigmoidal
cAMP	0.1	0.123		100	Hyperbolic
FDP[a]	0.1	0.20		100	Hyperbolic

[a] 3PGA, 3-phosphoglyceric acid; FDP, fructose-1,6-P_2.

this general set of phosphofructokinase properties are its specificity for substrates and cofactors as well as its sensitivity to citrate.

Thus, in common with other phosphofructokinases, both Mg^{2+} and Mn^{2+} are activating to the turtle heart enzyme, the optimal concentration of Mn^{2+} needed is lower than that for Mg^{2+}, and the V_{max} obtainable with Mn^{2+} is also lower (95). The K_m for manganese at pH 6.80 is so low, 5.8×10^{-6}M, that it might possibly play a role in <u>in vivo</u> enzyme function.

ADP is usually thought to be a deinhibitor of ATP inhibited phosphofructokinase (96) but at pH 7.90 it seems to serve to decrease the substrate specificity for the nucleoside triphosphate by increasing the affinity of the turtle enzyme for fructose-6-P when in the presence of GTP or ITP. Otherwise, the capacity of the enzyme to utilize ATP, GTP or ITP as phosphate donors does not appear to be unusual and has been previously reported for other phosphofructokinases (42).

Finally, a brief comment should be made concerning citrate effects on the enzyme. It is of course axiomatic that when oxygen is available in the organism's environment, the turtle heart displays a relatively normal aerobic metabolism. Under these conditions, citrate inhibition of glycolysis by blocking phosphofructokinase would in effect "spare" carbohydrate, the anaerobic energy store, and favor other substrates, particularly fatty acids, for aerobic metabolism. This role of citrate in phosphofructokinase control is entirely analagous to its accepted role in other, more oxygen dependent hearts (42,97).

Role of P-creatine in Control of Glycolytic Flux

The key role of P-creatine in controlling the *in vivo* kinetic behavior of turtle heart PFK prompted us to look at its effect in a well characterized mammalian enzyme (rabbit muscle) and ascertain how important and general this inhibitory effect is.

When vertebrate skeletal muscle is electrically stimulated under anoxic conditions, the glycolytic rate increases about 100 fold in a manner directly proportional to the stimulation frequency, and it can increase up to 600 fold during tetanus (98,99). Phosphofructokinase (EC 2.7.1.11) undoubtedly plays a pivotal role in this extreme swing from a low to a high rate of glycolysis,

and because the percent activation required during intense muscle work is so large, it is clear that this enzyme's regulatory properties must be geared to allow for essentially "complete on - complete off" catalytic behavior (100). How this may occur is not yet clear because the concentration of no single regulatory metabolite thus far studied falls or rises by a large enough factor to account for phosphofructokinase activation (98,99). One possible mechanism of activation calls for synergistic effects by various metabolites (101). Another possibility calls for mechanisms that amplify the small concentration changes in ATP and AMP. A third possibility is creatine phosphate.

Creatine phosphate displays a number of characteristics that warrant serious consideration in regard to glycolytic control. Firstly, it is already known to inhibit two glycolytic enzymes (102,61). Secondly, during large (100 fold) glycolytic activation in anoxic muscle, creatine phosphate concentrations drop from about 30 mM to 1 mM (99); at the same time, the concentration changes of other potentially important regulatory metabolites are very modest. ATP levels drop from 7.2 mM by only 15-30%; ADP concentrations increase from about 2.3 mM to 5.0 mM; AMP levels rise from 0.4 to 0.8 mM; inorganic phosphate levels rise from about 4 to 8 mM (98). Because both the percentage change and the absolute change in creatine phosphate concentrations far surpass those of other regulatory metabolites, any effect on a key enzyme such as phosphofructokinase would be reflected in large changes in glycolytic rate.

In agreement with previous studies (103), we found creatine phosphate to be a potent inhibitor of rabbit muscle phosphofructokinase. The inhibition is competitive with respect to both substrates, fructose-6-phosphate and ATP.

Dixon plots of 1/velocity versus creatine phosphate concentration indicate a K_I of about 13 mM with respect to fructose-6-phosphate. The effect of creatine phosphate plus a number of other phosphofructokinase modulators is shown in Figure 1. In common with other phosphofructokinases (42), fructose-6-phosphate saturation curves are strongly sigmoidal. Creatine phosphate greatly decreases the apparent enzyme-substrate affinity but has essentially no effect on the maximum velocity of the reaction. Most significantly, all creatine phosphate inhibitory effects can be fully reversed by AMP and inorganic phosphate, but the latter is clearly more effective. Thus in the presence of both inorganic phosphate and creatine phosphate, the substrate saturation curve becomes hyperbolic and there is a large (40 fold) increase in the apparent enzyme-substrate affinity as well as in the maximum velocity of the reaction. The combined regulatory effects of these metabolites raises the potential for sensitive control of this key step in glycolysis, but just how large might the role of creatine phosphate be?

To answer the latter question we measured the activity of muscle phosphofructokinase under conditions simulating muscle at rest and muscle at work (see 52 for similar attempts by others). From these experiments (Table 4), it is clear that the summed effects of increasing substrate availability, increasing positive modulator concentrations, and decreasing ATP lead to about a 100 fold activation of phosphofructokinase. When creatine phosphate concentrations are also dropped (from 30 to 1 mM), the overall phosphofructokinase activity rises by about 200 fold (Table 4). The magnitude of these effects therefore would appear to be large enough to account for the known percentage changes that occur in glycolysis during intense muscle work (101,102) but they would not appear to be large enough

TABLE 4

An attempt to assess the relative activities of phosphofructokinase under conditions simulating muscle at rest and muscle at work.

Rate relative to control[1]	CrP	ATP	ADP	AMP	P_i	G6P	F6P	NH_4^+	Mg^{2+}	K^+	Physiological state of muscle[2]
1	30	7.2	3.3	0.4	4.0	0.2	0.05	0.3	11	100	resting
100	30	4.0	4.0	0.8	8.0	1.9	1.0	1.0	11	100	creatine phosphate inhibited
200	1	4.0	4.0	0.8	8.0	1.9	1.0	1.0	11	100	contracting

[1]Control: The rate of reaction (0.0021 $O.D._{340}$/min) with the concentration of effectors and substrates observed in resting muscle.

[2]These values taken from (52).

to account for the level of glycolytic activation noted during extreme situations such as tetanus (98).

Clearly then, further investigation should proceed along two lines: (1) a study of the integration of glycolytic flux parameters in the anoxic heart by a kinetic appraisal of the other key control enzyme of glycolysis, PK, and (2) a study of creatine-P metabolism. The metabolism of FDP, the other key control metabolite, has already been investigated via the PFK study. Creatine-P levels must be controlled to allow for the rapid depletion seen in anoxia (4). The next chapter deals with pyruvate kinase, while Chapter 4 addresses itself to creatine kinase control.

PHOSPHOFRUCTOKINASE COMPARISON CHART

Parameters	Turtle Heart (103)	Sheep Heart (44)	Mammal Skeletal Muscle (42,91)	Brain (97)	Liver (91)
High pH	7.9	8.2	8.5	8.0	8.5
Kinetics	Hyperbolic	Hyperbolic	Hyperbolic	Hyperbolic	
K_m (in mM) Mg^{2+}	0.175	0.170	-	-	0.25
F6P	0.04	0.035	0.05	0.04	0.05
ATP	0.025	0.074	0.05	0.10	0.04
Low pH	6.8	6.9	7.0	7.1	7.4
Kinetics	Hyperbolic	Sigmoidal	Sigmoidal	Sigmoidal	Sigmoidal
K_m (in mM) F6P	0.06	Varies with [ATP]	0.06	0.27	0.13
ATP	0.02			0.02	
ATP Inhibition	a) with stabilizers: K_i = 8.5 mM b) without stabilizers: K_i = 6.3 mM	a) with stabilizers: K_i = 0.1 mM b) without stabilizers: K_i = 0.03 mM	K_i = 3.1 mM	Inhibition noted at 2 mM	K_i = 1.7 mM
Creatine Phosphate Inhibition	64% at 2.7 mM	Refractory	44% at 3 mM		

CHAPTER IV:

Pyruvate Kinase

The energetic inefficiency of glycolysis has been identified by several investigators as limiting in anaerobic survival where the heart and CNS must perform at zero or near-zero oxygen tensions (4,66,69).

Since previous studies identify phosphofructokinase and pyruvate kinase as important regulatory enzymes in glycolysis (23), our study was initiated by characterizing turtle heart phosphofructokinase and now pyruvate kinase.

We have found turtle heart pyruvate kinase to be an allosteric enzyme, but, unlike the mammalian homolog, to be profoundly affected by low concentrations of the feed forward activator, fructose diphosphate. Fructose-1,6-P_2 lowers the $K_{m(P\text{-enolpyruvate})}$ of the enzyme 5-fold and overrides ATP, phenylalanine and alanine inhibitions. The direct activation by fructose-1,6-P_2 of the turtle enzyme allows a more rapid generation of pyruvate and energy from anaerobic glycolysis while the complete reversal of ATP inhibition is important in a glycolytic tissue which displays no drop in ATP concentration during aerobic-anaerobic transition (35).

RESULTS

Electrofocusing

Electrophoretic resolution of pyruvate kinase activity in turtle heart shows a single band with pI 6.05. Rabbit skeletal muscle pyruvate kinase in 0.1 M P_i buffer exhibits a pI of 5.98 (105). After electrofocusing pyruvate kinase was dialyzed against imidazole buffer pH 7.5 containing 0.1 mM Mg^{2+}, 10 mM KCl, 20 mM β-mercaptoethanol to remove ampholines and sucrose and to stabilize the enzyme.

Cation Requirements

In common with pyruvate kinases from other species, turtle pyruvate kinase shows an absolute requirement for divalent and monovalent cations. Mg^{2+} or Mn^{2+} can satisfy the former requirement with a K_m for Mg^{2+} of 0.60 mM and K_m for Mn^{2+} of 0.133 mM. However, the V_{max} with Mn^{2+} is only 34% of that with Mg^{2+}. K^+ or NH_4^+ can satisfy the requirement for a monovalent cation (K_m of K^+ at pH 6.80 is approximately 11 mM).

pH Optimum and Effect of Fructose-1,6-P_2

The pH optimum of turtle heart pyruvate kinase is 6.3 with phosphate buffer and 6.55 with imidazole or triethanolamine. This is in contrast to the pH optimum of 8.5 for rabbit muscle pyruvate kinase (105). Fructose-1,6-P_2 activates at all pH's tested, but seems to give more activation at higher pH's. The enzyme shows quite a broad pH optimum which is further broadened by the addition of fructose-1,6-P_2.

Metabolite Effects

After prolonged anoxic work (12 hours) the intracellular pH of *P. scripta* heart drops to about 6.80 (4); therefore, this pH was chosen to study the catalytic and regulatory parameters of the enzyme.

TABLE I

Effects of metabolites on V_{max} of turtle heart pyruvate kinase

The basic assay mixture contained 20 mM imidazole, pH 6.80, 2 mM NADH, 0.7 mM ADP, 1.4 mM P-enolpyruvate, 5 mM Mg^{2+}, 60 mM KCl, and excess coupling enzyme. The reaction was started by the addition of the pyruvate kinase preparation.

Compound added	V_{max} (%)
None	100
1 mM 3-P-glyceric acid	150
1 mM fructose-6-P	117
1 mM glucose-1-P	110
0.1 mM fructose-1,6-P_2	107
1 mM creatine-P	106
0.5 mM AMP, cyclic AMP, serine	100
1 mM 2-P-glyceric acid	100
5 mM ATP	94
1 mM phenylalanine	78
1 mM alanine	54

Table I shows the effects of various metabolites on the V_{max} of the reaction at pH 6.8. 3P-glyceric acid yields the highest activation but is not effective in reversing ATP, alanine or phenylalanine inhibition. Phosphorylated hexoses activated pyruvate kinase slightly as did creatine phosphate in low concentration. Phenylalanine and especially alanine caused large decreases in the V_{max}.

Substrate Affinities

Michaelis constants were determined from Lineweaver-Burk plots and are 0.227 mM for ADP and 0.117 mM for P-enolpyruvate at pH 6.80 compared to 0.30 mM for ADP and 0.086 mM for P-enolpyruvate at pH 8.50 for rabbit skeletal muscle (58). GDP and IDP are found to substitute for ADP, both exhibiting sigmoidal saturation curves. In the presence of GDP, the value of $[S]_{0.5}$ is 0.32 mM, the value of n is 2.0, and the enzyme achieves 24% of the V_{max} observed in the presence of ADP. With IDP as the cosubstrate, the value of $[S]_{0.5}$ is 0.56 mM, the Hill coefficient is 1.7, and the enzyme achieves 21% of the V_{max} observed with ADP as cosubstrate. With GDP or IDP as cosubstrates, maximum velocities of the mammalian muscle enzyme are 60% and 75% respectively, of the V_{max} obtainable with ADP (58).

The Mn^{2+} activated enzyme shows a much lower $K_{m(ADP)}$ of 0.057 mM but the $K_{m(P\text{-enolpyruvate})}$ does not change and the maximal velocity is only 34% of the Mg^{2+} activated enzyme.

The K_m of either substrate is unaffected by varying the concentration of the other. The K_m for P-enolpyruvate is lowered drastically from 0.117 mM to 0.021 mM (almost 6 fold) by the addition of 0.1 mM fructose-1,6-P_2. By comparison the K_m of ADP is unaffected by the addition of fructose-1,6-P_2.

"V_{max}" Inhibition

The concentrations of various inhibitors of pyruvate kinase were varied to test their effect on the enzyme under saturating conditions of substrates (see Materials and Methods).

Figure 1 shows the effect of increasing phenylalanine concentration from zero to 10 mM. Serine deinhibits to some extent, increasing the phenylalanine concentration necessary to achieve 50% inhibition from 2 mM to 6.5 mM and by lowering the n value from 2.80 to 2.08. Fructose-1,6-P_2 is a more potent deinhibitor and lowers the n value to 1.50.

Alanine is a more potent inhibitor of pyruvate kinase than phenylalanine with 77% inhibition at 1 mM alanine. Fructose-6-P provides little protection against inhibition whereas 0.1 mM fructose-1,6-P_2 almost completely reverses alanine inhibition at all concentrations tested (Figure 2).

ATP is a less potent inhibitor with 50% inhibition occurring at 5 mM. Fructose-1,6-P_2 (at 0.1 mM) and serine (at 0.6 mM) reverse this inhibition somewhat with 50% inhibition at 10 mM and 7 mM ATP respectively (Figure 3).

Creatine-P showed almost no inhibition of pyruvate kinase activity up to 10 mM with 50% inhibition not reached until 20 mM. For every inhibitor used the following set of compounds were tried as deinhibitors, and if not specifically mentioned were found ineffective: 1 mM fructose-6-P, glucose-1-P, 3P-glyceric acid, 2P-glyceric acid; 0.5 mM AMP; 0.1 mM cAMP.

Nature of ATP Inhibition

In common with pyruvate kinases from other sources (106,107), ATP inhibits turtle heart pyruvate kinase. The K_i for ATP with respect to ADP is 3.70 mM and the inhibition is noncompetitive (Figure 4).

Figure 1. Phenylalanine inhibition of turtle heart pyruvate kinase at pH 6.8 and its reversal by 0.6 mM serine and 0.1 mM fructose-1,6-P_2. Reaction concentrations are: 20 mM imidazole buffer, pH 6.8; 5 mM Mg^{2+}, 60 mM K^{+}, 0.7 mM ADP, 1.4 mM P-enolpyruvate, 0.2 mM NADH, excess lactate dehydrogenase, and increasing concentrations of phenylalanine. Additions are: Θ, none; □ , 0.6 mM serine; Δ, 0.1 mM fructose-1,6-P_2.

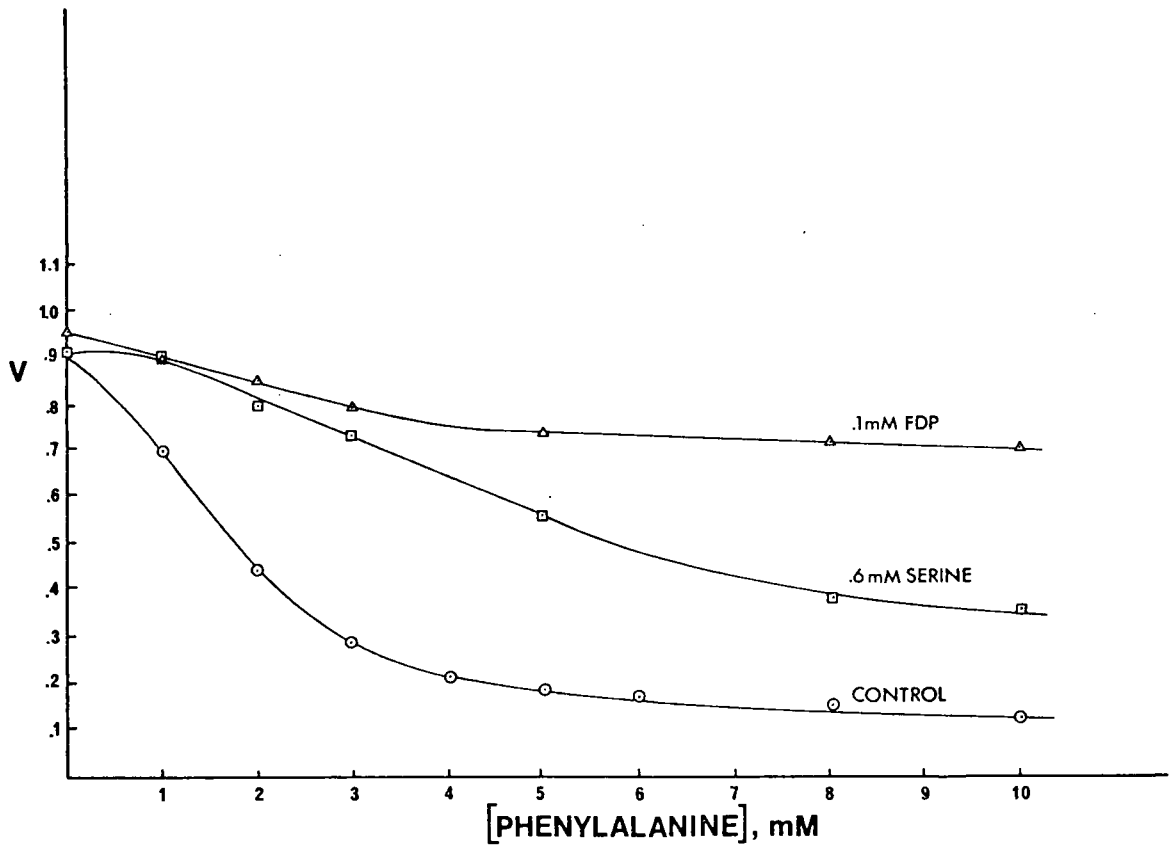

V
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1
2
3
4
5
6
7
8
9
10
.1mM FDP
.6 mM SERINE
CONTROL
[PHENYLALANINE], mM

Figure 2. Alanine inhibition of turtle heart pyruvate kinase at pH 6.8 and its reversal by 1 mM fructose-6-P and 0.1 mM fructose-1,6 -P_2. Reaction conditions are the same as in Fig. 1, here with increasing alanine concentrations as the variable. Additions are: Δ , none; ⊡ , 1 mM fructose-6-P; ⊖, 0.1 mM fructose-1,6-P_2.

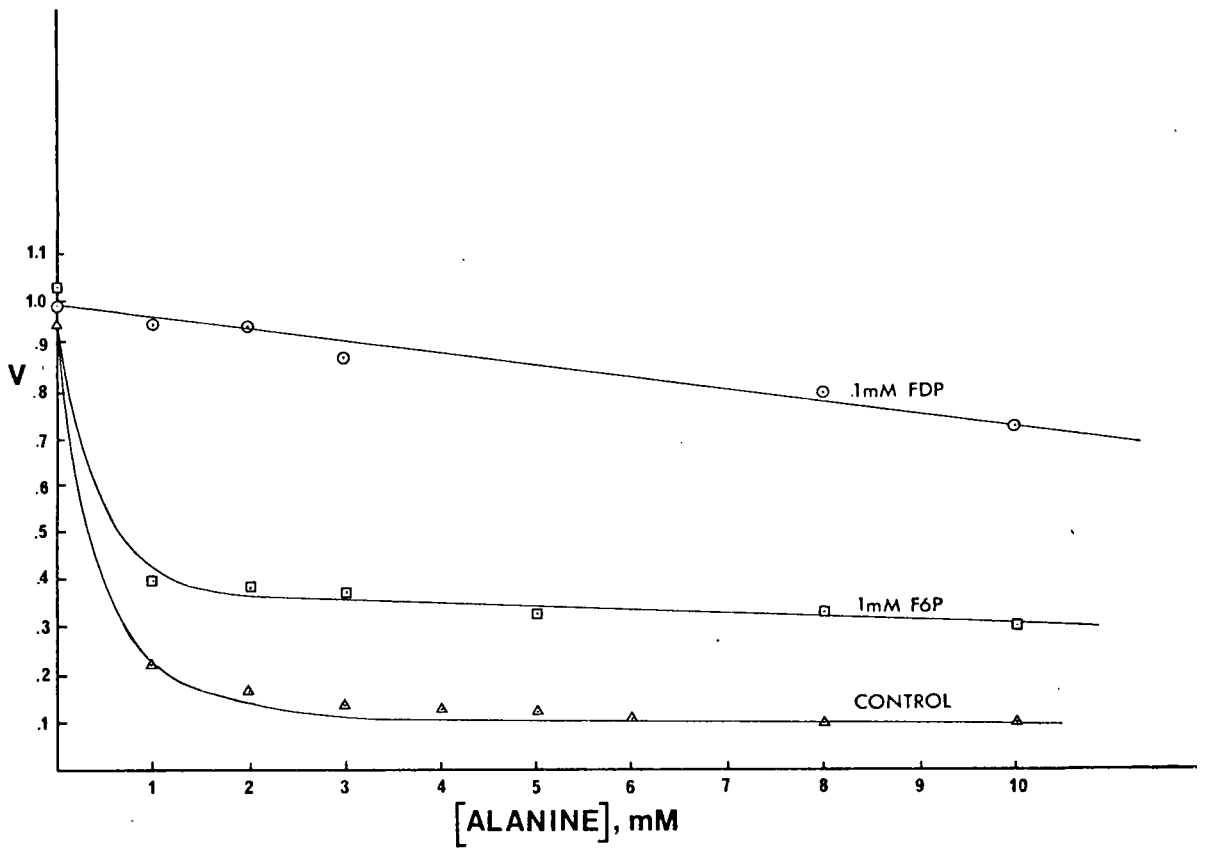

V
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1mM FDP
1mM F6P
CONTROL
1
2
3
4
5
6
7
8
9
10
[ALANINE], mM

Figure 3. MgATP inhibition of turtle heart pyruvate kinase at pH 6.8 and its reversal by 0.6 mM serine and 0.1 mM fructose-1,6-P_2. Reaction concentrations are the same as for Fig. 2, here with increasing MgATP concentrations as the variable. Additions are: ●, none; Δ , 0.6 mM serine; ⊡ , 0.1 mM fructose-1,6-P_2.

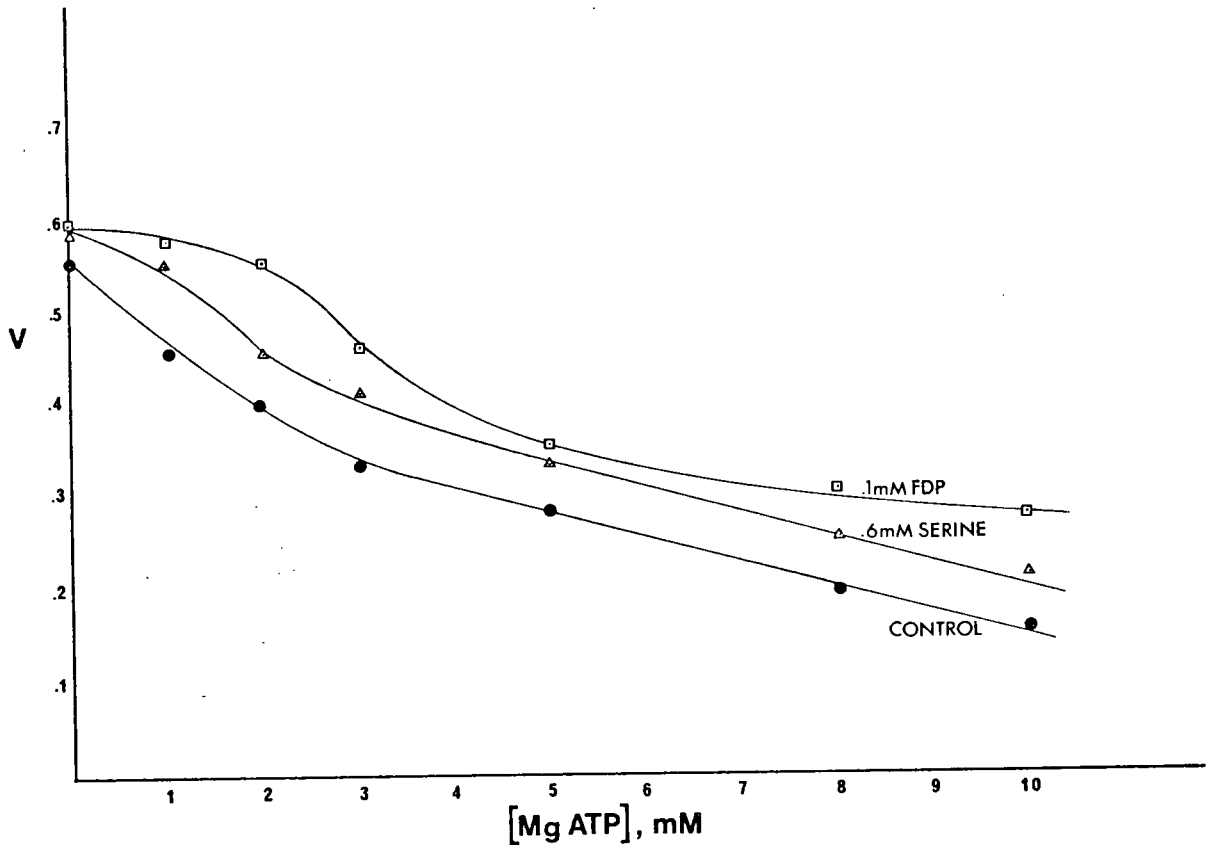

.7
.6
.5
.4
.3
.2
.1
V
.1mM FDP
.6mM SERINE
CONTROL
1
2
3
4
5
6
7
8
9
10
[Mg ATP], mM

Figure 4. Double reciprocal plot of the reaction velocity of turtle heart pyruvate kinase, pH 6.8, at different ATP concentrations. Reaction concentrations are: 20 mM imidazole buffer (pH 6.8), 5 mM Mg^{2+}, 60 mM K^{+}, 1.4 mM P-enolpyruvate, increasing concentrations of ADP, ATP concentrations (●, no ATP; □ , 1 mM ATP; Δ 2 mM ATP; ○, 3 mM ATP), and excess lactate dehydrogenase.

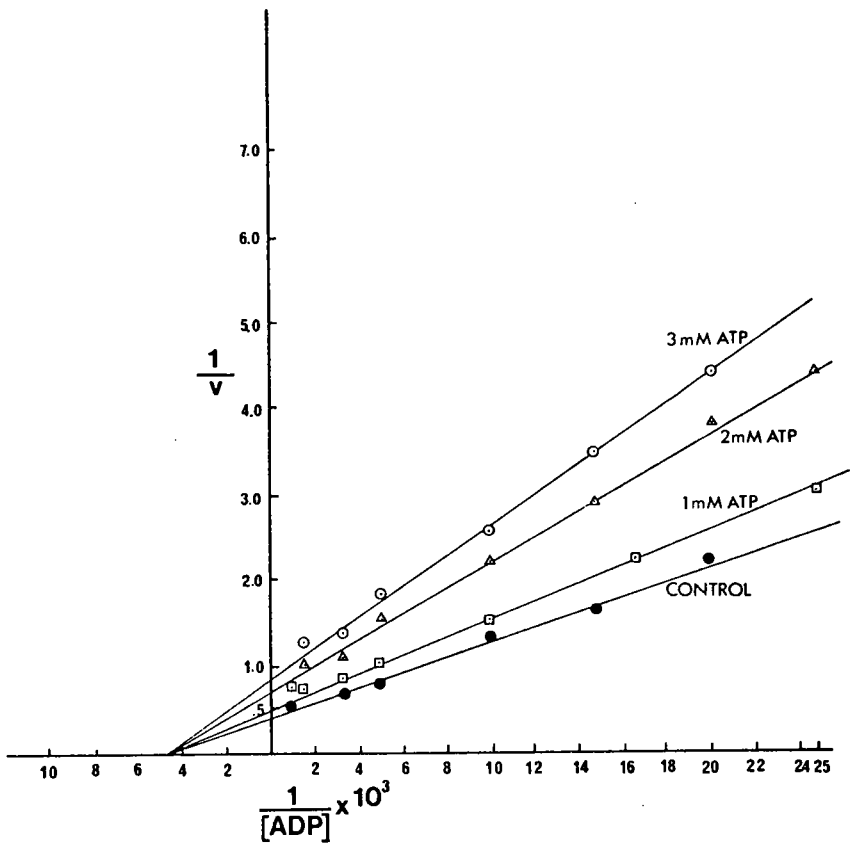

7.0
6.0
5.0
4.0
3.0
2.0
1.0
.5
1/V
3mM ATP
2mM ATP
1mM ATP
CONTROL
10 8 6 4 2
2 4 6 8 10 12 14 16 18 20 22 24 25
1/[ADP] x 10³

Figure 5 shows that ATP inhibition is mixed competitive with respect to P-enolpyruvate. The inhibition was found to be of the partially competitive type where the inhibitor affects the affinity of the enzyme for the substrate although the inhibitor and substrate combine at different groups. Dixon plots of 1/V vs inhibitor concentration do not give a straight line, as the effective affinity of the enzyme for the inhibitor varies with the substrate concentrations at each value.

ATP inhibition was reversed by fructose-1,6-P_2 and AMP. The K_m of P-enolpyruvate rose from 0.117 mM to 0.56 mM in the presence of 3 mM ATP. With 0.1 mM cAMP the K_m dropped to 0.345 mM whereas 0.1 mM fructose-1,6-P_2 plus 3 mM ATP gave a $K_{m(\text{P-enolpyruvate})}$ of 0.095 mM (Figure 6).

Nature of Creatine-P Inhibition

The K_i with respect to P-enolpyruvate for creatine-P is 2.2 mM and is seen to be competitive by Dixon plot, whereas the K_i with respect to ADP is 19.6 mM and noncompetitive. None of the various metabolites tested (see above) were effective in reversing creatine-P inhibition. Compensation for Mg^{2+} chelation was also ineffective in reversing the inhibitory effects. Creatine-P (at 2 mM) raises the K_m for P-enolpyruvate from 0.117 mM to 0.182 mM.

Nature of Alanine and Phenylalanine Inhibition

Phenylalanine and alanine are known to inhibit muscle type pyruvate kinase in a manner competitive with respect to P-enolpyruvate (108-111). For the turtle heart enzyme this is also true with the K_i values being 0.35 mM for phenylalanine and 0.09 mM for alanine. As in the case of ATP inhibition, fructose-1,6-P_2 not only releases the inhibition but overrides it. In the presence of 3 mM phenylalanine the K_m for

Figure 5. Double reciprocal plot of the reaction velocity of turtle heart pyruvate kinase at pH 6.8. Reaction concentrations are: 20 mM imidazole (pH 6.8), 5 mM Mg^{2+}, 60 mM K^{+}, 0.4 mM ADP, increasing concentrations of P-enolpyruvate (PEP), ATP concentrations (○, no ATP; □, 1 mM ATP; △, 2 mM ATP; ●, 3 mM ATP), and excess lactate dehydrogenase.

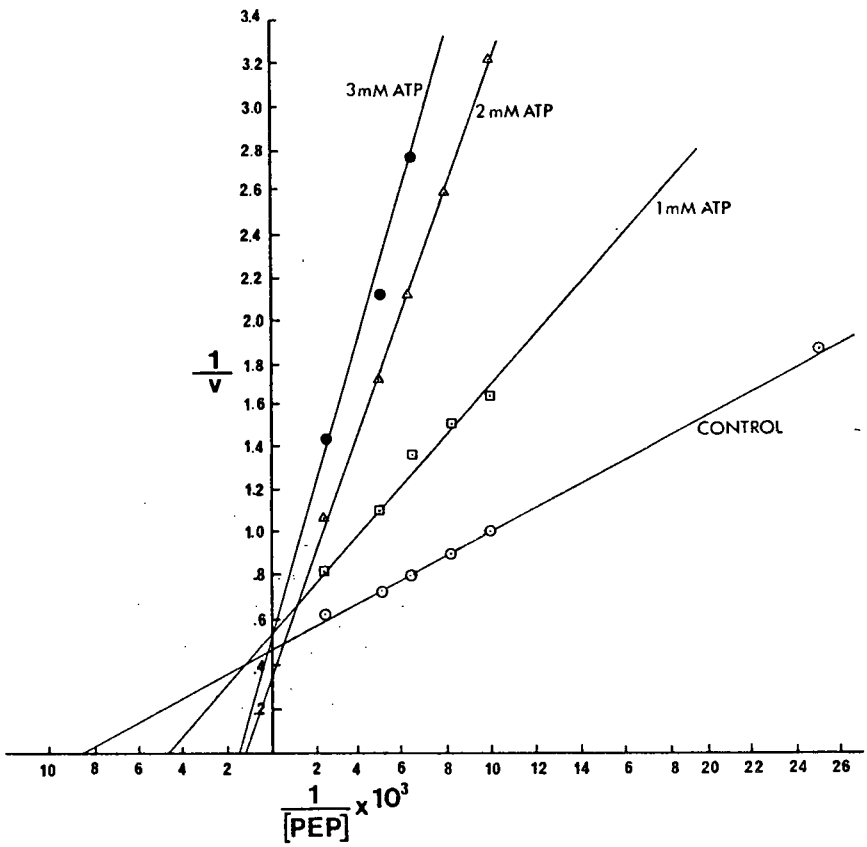
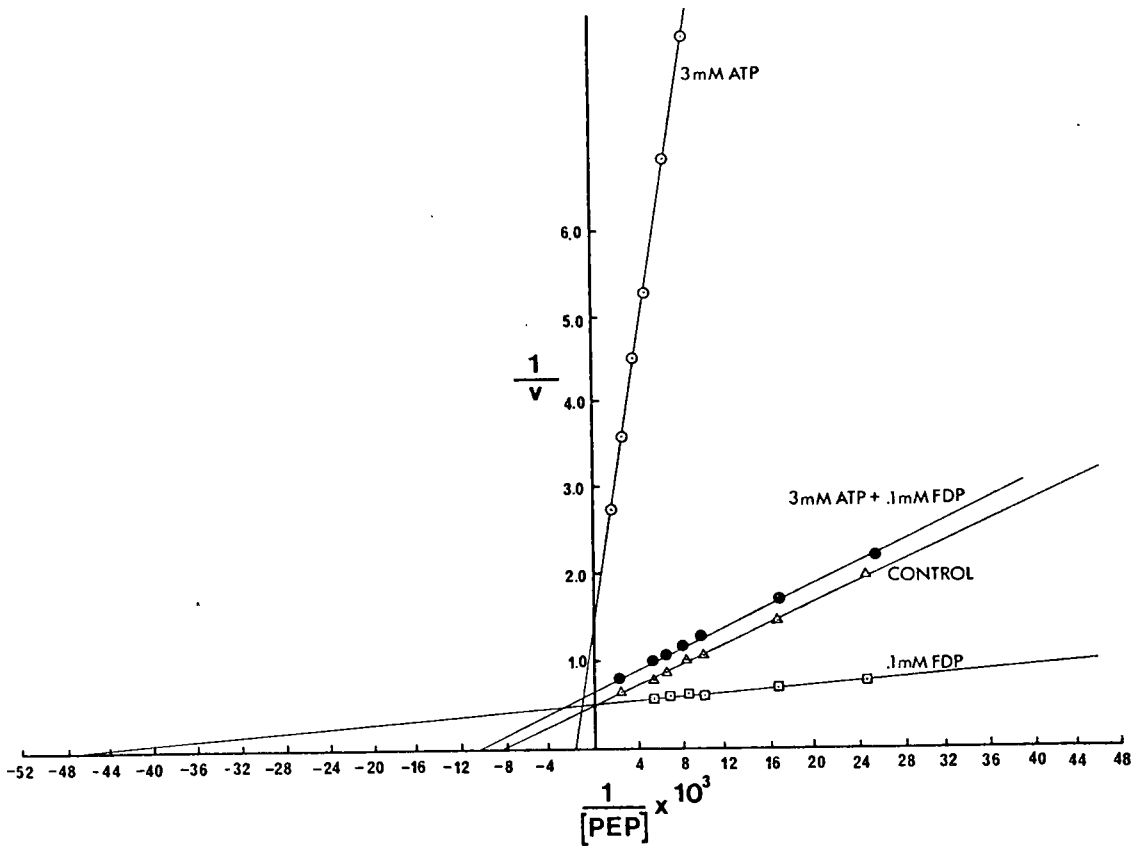

3mM ATP
2 mM ATP
1mM ATP
CONTROL
1/V
1/[PEP] x 10³
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
10 8 6 4 2 2 4 6 8 10 12 14 6 8 20 22 24 26

Figure 6. Interacting effects of ATP and fructose-1,6-P_2 on the $K_{m(P\text{-enolpyruvate})}$ for turtle heart pyruvate kinase at pH 6.8. Reaction concentrations are the same as in Fig. 5, but 3 mM ATP and 0.1 mM fructose-1,6-P_2 were added as indicated. Additions are: Δ , none; ⊡ , 0.1 mM fructose-1,6-P_2; ○, 3 mM ATP; ●, 3 mM ATP plus 0.1 mM fructose-1,6-P_2. PEP, P-enolpyruvate.


3mM ATP
3mM ATP + .1mM FDP
CONTROL
.1mM FDP
1/v
6.0
5.0
4.0
3.0
2.0
1.0
-52 -48 -44 -40 -36 -32 -28 -24 -20 -16 -12 -8 -4
4 8 12 16 20 24 28 32 36 40 44 48
1/[PEP] x 10^3

P-enolpyruvate rises from 0.117 mM to 1.33 mM but 0.1 mM fructose-1,6-P_2 reduces the K_m to 0.042 mM. Alanine (at 3 mM) also raises the $K_{m(P\text{-enolpyruvate})}$ to 0.33 mM and 0.1 mM fructose-1,6-P_2 again reduces this to 0.042 mM (Figure 7).

Other Inhibitors

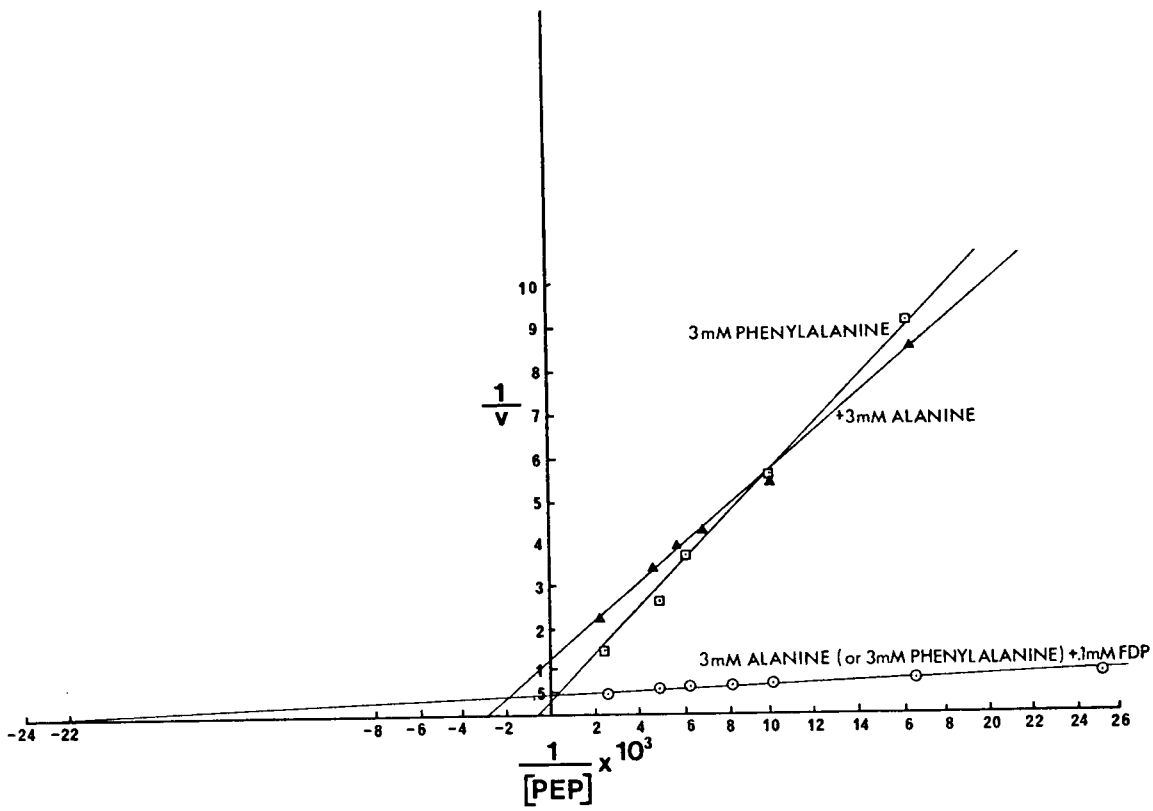
Calcium, known to inhibit muscle pyruvate kinase (58), inhibits the turtle enzyme noncompetitively with respect to ADP and P-enolpyruvate with a K_i value of 1 mM. Citrate, also an inhibitor, inhibits turtle heart pyruvate kinase competitively with respect to P-enolpyruvate with a K_i of 1.65 mM. Like their mammalian counterparts, turtle hearts exhibit normal aerobic metabolism when oxygen becomes available and citrate inhibition of glycolysis at the pyruvate kinase step probably serves with citrate inhibition of phosphofructokinase (91) to "spare" carbohydrate. To facilitate comparison between the turtle heart enzyme and other pyruvate kinases a chart is provided (see page 87).

DISCUSSION

The data in this study suggest that the turtle heart enzyme differs in several ways from mammalian muscle pyruvate kinase and that these differences probably reflect the need for different control mechanisms.

The pH optimum of the turtle heart enzyme is 2 pH units below that of the mammalian muscle enzyme and could reflect the need for a high glycolytic flux in the face of increasing tissue acidity during the anaerobic production of large amounts of lactate (4). The Michaelis constants for ADP are similar for mammalian muscle and turtle heart pyruvate kinases but the $K_{m(P\text{-enolpyruvate})}$ is nearly 1.5 times larger

Figure 7. Interacting effects of phenylalanine or alanine and fructose-1,6-P_2 on the $K_{m(P\text{-enolpyruvate})}$ for turtle heart enzyme at pH 6.8. Reaction conditions are the same as in Fig. 6, but 3 mM alanine, 3 mM phenylalanine, and 0.1 mM fructose-1,6-P_2 were added as indicated. Additions are: ▲ , 3 mM alanine; ⊡ , 3 mM phenylalanine; 0, 3 mM alanine plus 0.1 mM fructose-1,6-P_2 or 3 mM phenylalanine plus 0.1 mM fructose-1,6-P_2. PEP, P-enolpyruvate.


3mM PHENYLALANINE
+3mM ALANINE
3mM ALANINE (or 3mM PHENYLALANINE) +.1mM FDP
1/V
1/[PEP] x 10³

PYRUVATE KINASE COMPARISON CHART

Parameter	Turtle Heart Enzyme (103)	Mammalian Muscle Enzyme (109)	Oyster Adductor Enzyme (8)
K_m in mM			
pH 6.80			
PEP	0.117	0.086	0.08
PEP & FDP	0.021	same	0.04
ADP	0.23	0.30	0.10
Mg^{2+}	0.60	-	2.2
K^+	11	12	0.77
pH opt	6.5	8.5	8.5
FDP effect	feed forward activator	no known effects	feed forward activator
pI	6.05	5.98	5.6; 6.5
ATP Inhibition	mixed competitive	competitive	competitive
	FDP reverses and overrides	no FDP effect	FDP reverses and overrides
K_i	2.1 mM	17 mM	2.8 mM
Alanine Inhibition	competitive	competitive	mixed competitive
	FDP reverses and overrides	no FDP effect	FDP reverses and overrides
K_i	0.09 mM	20 mM	2.7 mM
Phenylalanine Inhibition	competitive	mixed competitive	mixed competitive
	FDP reverses and overrides	no FDP effect	FDP reverses and overrides
K_i	0.35 mM	11 mM	3.8 mM
Calcium Inhibition K_i	1 mM	2-3 mM	
Citrate $(Mg)_2$ Inhibition K_i	1.65 mM	no known effects	
Creatine Phosphate Inhibition	50% at 20 mM	70% at 20 mM	
2 PGA	no effect	isosteric inhibitor	

for the turtle enzyme. However, in the presence of fructose-1,6-P_2 the $K_{m(P\text{-enolpyruvate})}$ for the turtle enzyme drops to 0.021 mM, fully four fold lower than for the enzyme in mammalian muscle. This strong activation by fructose-1,6-P_2 (causing a large decrease in the $K_{m(P\text{-enolpyruvate})}$) may reflect a physiological mechanism whereby pyruvate kinase activity can be increased drastically during times when the phosphofructokinase step is activated, that is, during the phase which is characterized by an increasing glycolytic flux (112,113). This feed forward activation by fructose-1,6-P_2 has been observed for sea mussel (Mytilus edulis L.) adductor muscle (114) and oyster adductor muscle (115), both of which must operate for long periods under anaerobic conditions.

The turtle heart also appears to be under tight ATP regulation. The $K_{i(ATP)}$ is about 1/4 the value for the enzyme from mammalian muscle (116). At 2 mM ATP, a value probably within the physiological range (117), this reaction product causes a 5 fold increase in the $K_{m(P\text{-enolpyruvate})}$. Under conditions of low P-enolpyruvate concentrations, it therefore is evident that turtle heart pyruvate kinase would be unusually sensitive to ATP, and this control presumably would be of importance in "turning off" glycolysis when aerobic conditions return and the heart preferentially utilizes other substrates. However, it is evident that some mechanism besides dropping ATP concentrations must exist for reversing ATP inhibition of the enzyme because (1) glycolysis is clearly activated during aerobic-anaerobic transition, but (2) ATP concentrations do not change at this time (35).

At least two compounds, fructose-1,6-P_2 and cAMP, are capable of reversing ATP inhibition of turtle heart pyruvate kinase, and of these, the fructose-1,6-P_2 reversal is considered the more important because cAMP

concentrations required are 2-3 orders of magnitude higher than probable in vivo levels (93). Moreover, at concentrations well within the expected physiological range (118) fructose-1,6-P_2 leads (a) to a complete reversal of ATP inhibition of pyruvate kinase, (b) to a direct increase in the apparent enzyme affinity for substrate, and (c) to an integration of pyruvate kinase deinhibition with phosphofructokinase activation (103). These characteristics are the hallmarks of an excellent modulator and suggest a particularly pivotal role for fructose-1,6-P_2 in the control of the high glycolytic capacity of turtle myocardium.

Mammalian muscle enzyme and turtle heart enzyme also seem to differ in the effects of other inhibitors. For both enzymes alanine is a competitive inhibitor but the K_i values differ drastically, being 20 mM for the mammalian (59) and 0.09 mM for the turtle pyruvate kinase. Although fructose-1,6-P_2 has no effect on the mammalian enzyme, the $K_{m(P\text{-enolpyruvate})}$ of turtle pyruvate kinase decreased 9 fold on addition of 0.1 mM fructose-1,6-P_2 to the alanine-inhibited enzyme. Phenylalanine is a mixed competitive inhibitor of the mammalian enzyme with a K_i (at 0.6 mM P-enolpyruvate) of 11 mM and no reversal of inhibition is seen with fructose-1,6-P_2 (59). For the turtle enzyme phenylalanine is a competitive inhibitor (with respect to P-enolpyruvate) with a K_i of 0.35 mM. The apparent K_m for P-enolpyruvate changes 26 fold by the addition of 0.1 mM fructose-1,6-P_2 to the phenylalanine-inhibited enzyme. Creatine-P has very little effect on turtle heart pyruvate kinase in contrast to its role in phosphofructokinase control from this tissue (103).

2P-glyceric acid, an analogue of P-enolpyruvate and an isosteric inhibitor of mammalian muscle pyruvate kinase (59) strangely shows no effect on the turtle enzyme even in concentrations up to 8 mM.

CHAPTER V:

Creatine Kinase

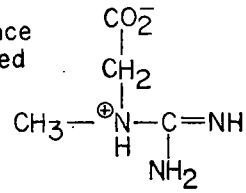
Creatine kinase (EC 2.7.3.2) catalyzes the reaction:

$$MgATP^{-2} + creatine^{\pm} \rightleftharpoons MgADP^{-} + phosphocreatine^{-2} + H^{+}$$

with the reaction proceeding from right to left designated as the forward reaction.

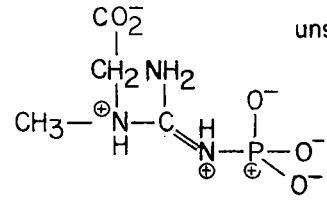
CREATINE

resonance stabilized



CREATINE-P

unstable due to repulsion



The enzyme is a dimer (119) with a muscle form MM, a brain form BB, and an intermediate form MB. Mammalian heart extracts usually contain largely the "muscle enzyme" and occasionally the intermediate enzyme.

In ox and rat brain creatine kinase activity was found to be equally partitioned between cytosol and the mitochondria (120) and both enzymes had similar properties (121,122). The properties of the enzymes from ox brain (123) are similar to those of the enzyme from rabbit muscle (124). The pH optimum for the forward direction is 7.0 while that for the reverse is 9.2. Noda et al (125) have shown that the apparent K_m values for the muscle enzyme are not appreciably affected by pH over the range of 6.4-8.9 whereas the V_{max} changes with change in pH.

The mammalian muscle enzyme is easily purified by virtue of its great stability in high ethanol concentrations plus heat (42°C) and alkaline pH (8.5).

Control of Creatine Kinase (CK)

Although CK is thought to be an equilibrium enzyme (affected by supply of substrates) and no effectors of CK have been found, several observations point

to control possibilities: (a) Although large pH changes affect CK equilibrium and the decline in muscle intracellular pH has been cited as the major control of the production of ATP by the enzyme, Kuby et al (124) while studying Mg^{2+} effects found that almost no change in $K_{equil.}$ was induced by a change of 0.1 to 0.2 of a pH unit. The intracellular pH of turtle heart drops about 0.2 of a unit in 12 hours of anoxic function and much less than 0.1 of a unit in the first hours when creatine phosphate utilization is the greatest. (b) A large discrepancy exists in the CK equilibrium in muscles. For the purified enzyme $K_{equil.}$ = 0.01 at pH 7.4 and 30°C. For abdominal wall muscle of rats a value of 0.23 has been reported (126). Helmrich and Cori (98)working with resting frog sartorii muscle have found the in vivo value of $K_{equil.}$ to be 0.32. The enzyme in vivo seems to be "held" from producing ATP as compared to the in vitro situation. (c) Although creatine-P is considered a tissue energy store to be utilized in emergencies, its function in turtle heart is perhaps different. Blix (74) found that diving and nondiving animals had the same creatine content of brain, hearts and skeletal muscles with heart values being 15-18 micro moles/gm tissue. This finding suggests that creatine-P is not an important energy source peculiar to diving animals. Turtle heart levels of creatine-P are about 1/5 that of mammals (3.4 μmoles/gm). This low creatine-P concentration in turtle heart casts doubt on its function as a store of energy in anaerobiosis because other sources of anaerobic energy (glycogenolysis) in the turtle heart have shown adaptations toward greater production not a dwindling of anaerobically useful energy reserves. If creatine phosphate was an important anaerobic energy supply its levels (like those of glycogen) could be expected to be at least as large as those of mammals. Creatine-P in turtle hearts could

function mainly as a signal for glycolysis in the on-off functioning of PFK. Glycolysis in turtle heart would then be regulated by the "energy status" of the cell as opposed to the energy charge. The hydrolysis of creatine-P as an energy source could be left to an unmodulated equilibrium enzyme, i.e. low levels of ATP signal the break up of storage energy but if the major function of creatine-P is the control of energy pathways (analogous to ATP elsewhere) its rates of synthesis and breakdown will be as rigidly controlled as those of ATP in other hearts.

Lehninger (127), working on CK in rat heart mitochondria, has postulated that this form of the enzyme is involved in smoothing out or buffering the rate of respiration of heart mitochondria at a high and constant rate as well as to channel the high energy phosphate to the contractile system. This stimulation of respiration by creatine is due to the continuous regeneration of ADP brought about by the rapid transfer of the terminal phosphate group of ATP to creatine by CK. When creatine in in vivo concentrations (10 mM) is added to heart mitochondria with ADP a rapid change from State 4 to State 3 respiration occurs - much more than with ADP alone.

Creatine and creatine-P are known to have several potentially critical roles in skeletal muscle (128). With creatine kinase, creatine-P participates in a system that regenerates ATP during contraction as well as inhibiting phosphofructokinase, pyruvate kinase, and glyceraldhyde 3-P dehydrogenase and activating FDPase. Creatine-P has been shown to be the major negative modulator of turtle heart glycolysis (103) due to its specific inhibitory effect on the phosphofructokinase reaction. During anoxic stress creatine-P levels in turtle heart fall allowing glycolytic flux to greatly increase (4).

Very low concentratoions of NADH (0.04 mM) are seen to activate creatine kinase in the creatine-P hydrolysing direction by lowering the K_m for creatine-P. This coupled with the knowledge that anoxic turtle tissues fall out of redox balance quickly and build up NADH (129) points to a redox based control of creatine-P levels which in turn control glycolytic flux.

RESULTS

Electrophoretic Studies

Electrofocussing of a high speed supernatant or a 55% to 65% ammonium sulfate cut of turtle heart reveals two major bands of creatine kinase activity with isoelectric points of 6.67 and 7.37 (Figure 1). These 2 peaks correspond to the 2 heart bands found by other investigators (52,119), and are thought to correspond to the BB or brain isozyme and the MB hybrid. After electrofocusing the creatine kinase isozymes were separated, pooled separately and run on starch gels where it was seen that each was essentially free of the other. After collection each form was dialyzed against imidazole buffer pH 7.5 containing 20 mM β-mercaptoethanol to remove ampholines and sucrose and stabilize the enzyme.

Figure 2 shows the creatine kinase electrophoretic patterns obtained on starch gels. In common with other animals (119) turtle muscle shows one isozyme (designated MM), and the brain shows two other, faster migrating, forms (one BB and the other thought to be a hybrid MB). The heart contains mainly the brain pattern with some of the muscle isozyme visible. Figure 2 also shows that when isolated mitochondria from heart were run only the slower major isozyme (MB) was present, indicating that this is the mitochondrial form and that the other isozyme is confined to the cytosol.

Figure 1. Electrofocusing run of a 55% to 65% ammonium sulfate cut of turtle heart assayed for creatine kinase. The electrophoresis was run as outlined in Materials and Methods. Assay reactant concentrations are: 20 mM imidazole buffer pH 6.7, 5 mM $Mg(Acetate)_2$, 1 mM ADP, 5 mM creatine-P, 0.2 mM NADP, 4 mM glucose and excess dialyzed Sigma HK and G6PDH. Fractions of one ml were collected.

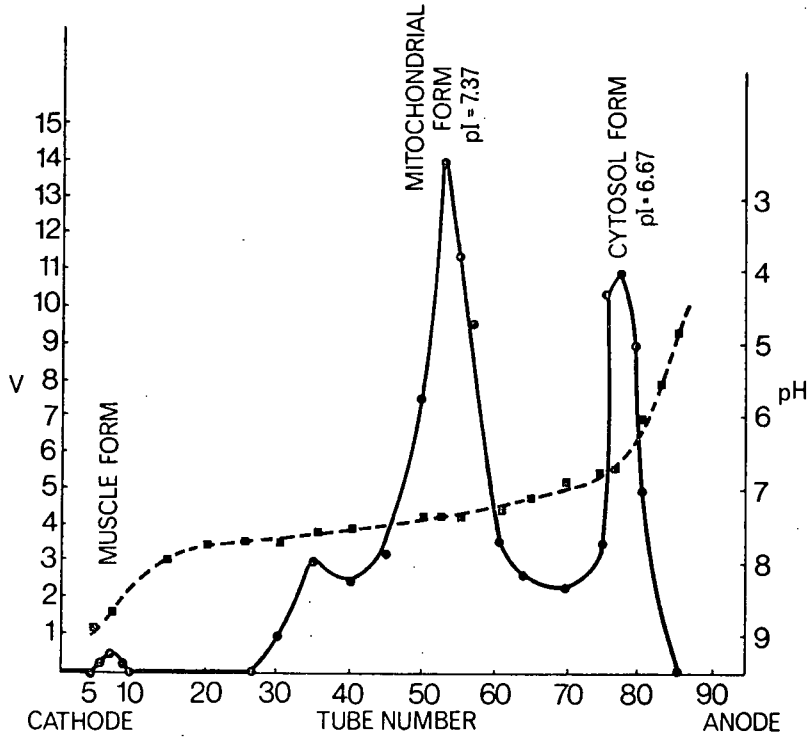
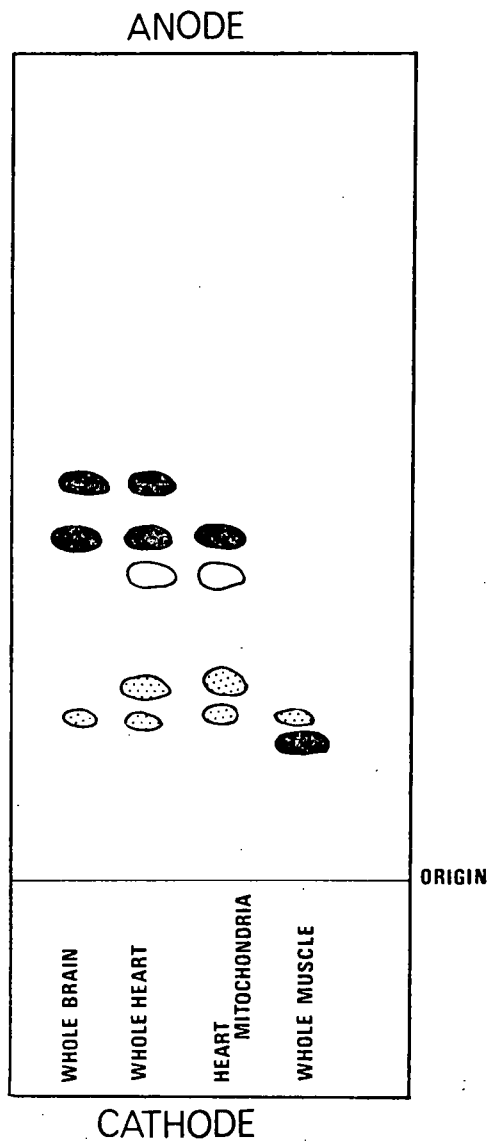

MITOCHONDRIAL FORM
pI = 7.37
CYTOSOL FORM
pI = 6.67
MUSCLE FORM
V
pH
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
3
4
5
6
7
8
9
5 10 20 30 40 50 60 70 80 90
CATHODE
TUBE NUMBER
ANODE

Figure 2. Electrophoretic patterns obtained with crude extracts and heart mitochondrial preparations (see Materials and Methods) of heart, skeletal muscle and brain from the adult turtle Pseudemys scripta. The staining medium consisted of Tris buffer, 0.1 M, pH 7.5, 15 ml; $MgCl_2$, 0.1 M, 1.0 ml; glucose, 0.1 M, 1.0 ml; excess HK and G6PDH; creatine-P, 0.1 M, 0.6 ml; ADP, 0.05 M, 0.5 ml; NADP, 0.035 M, 0.5 ml; Nitro BT, 10 mg/ml, 1.0 ml; and PMS, 1 mg/ml, 0.12 ml. In crude extracts of these tissues, adenylic kinase is also visualized by this method (indicated by hatched areas); it can be partially inhibited by the addition of AMP, 0.1 M, 1.0 ml, to the staining medium. Filled spots represent heavy stain; empty ones represent lighter staining.


ANODE
ORIGIN
WHOLE BRAIN
WHOLE HEART
HEART MITOCHONDRIA
WHOLE MUSCLE
CATHODE

pH Profiles

The cytosol isozyme has a pH optimum between 6.6 and 6.8 in the forward direction which is in agreement with optimal pH 6.7 values from a number of different species (127). The mitochondrial form displays a very broad optimum from 5.7 to about 6.1 which is lower than the reported value for rat heart enzyme of 6.7 (127). The optimum for the mitochondrial enzyme in the opposite direction is about pH 9.0, compared to a previously reported value of pH 8.0 (127).

Substrate Affinities

Cytosol enzyme: Michaelis constants at pH 6.7 are 0.33 mM for Mg^{2+}, 1.25 mM for creatine-P, and 0.045 mM for ADP compared to 3.6 mM for creatine-P and 0.12 mM for ADP from beef heart cytosol enzyme (127). The K_m of either substrate is unaffected by varying the concentration of the other. The K_m for creatine-P is lowered from 1.25 mM to 0.5 mM (a drop of nearly 3-fold) by the addition of 0.12 mM NADH (Figure 3).

Mitochondrial enzyme: Michaelis constants are 0.71 mM for Mg^{2+}, 0.57 mM for creatine-P, and 0.022 mM for ADP at pH 6.7 compared to 0.72 mM for creatine-P and 0.035 mM for ADP for beef heart mitochondrial enzyme (127). Figure 4 shows NADH to be an inhibitor of the mitochondrial enzyme with a K_i of 0.1 mM non-competitive with either substrate.

Reverse direction: At pH 9.0 the mitochondrial enzyme exhibits a K_m for creatine of 19 mM compared to 6 mM for the rat heart enzyme. The K_m for ATP for the turtle mitochondrial form is 0.6 mM compared to 0.1 mM for the rat heart enzyme. It was impossible to reverse the cytosol isozyme to any appreciable degree under these conditions. The beef heart cytosol isozyme (127) displays Michaelis constants for creatine of 33.3 mM and for ATP of

Figure 3. Double reciprocal plot of the effect of NADH on creatine-P saturation kinetics of the cytosol enzyme. Reaction concentrations are: 20 mM imidazole buffer, pH 6.7, 5 mM $Mg(Acetate)_2$, 0.8 mM ADP, 0.2 mM NADP, 4 mM glucose, excess dialyzed Sigma HK and G6PDH, varying creatine-P concentrations and NADH at the concentrations below: 0, no added NADH; Δ, plus 0.06 mM NADH; ⊡, plus 0.12 mM NADH. Reaction velocity (V) is expressed in terms of A_{340} per minute.

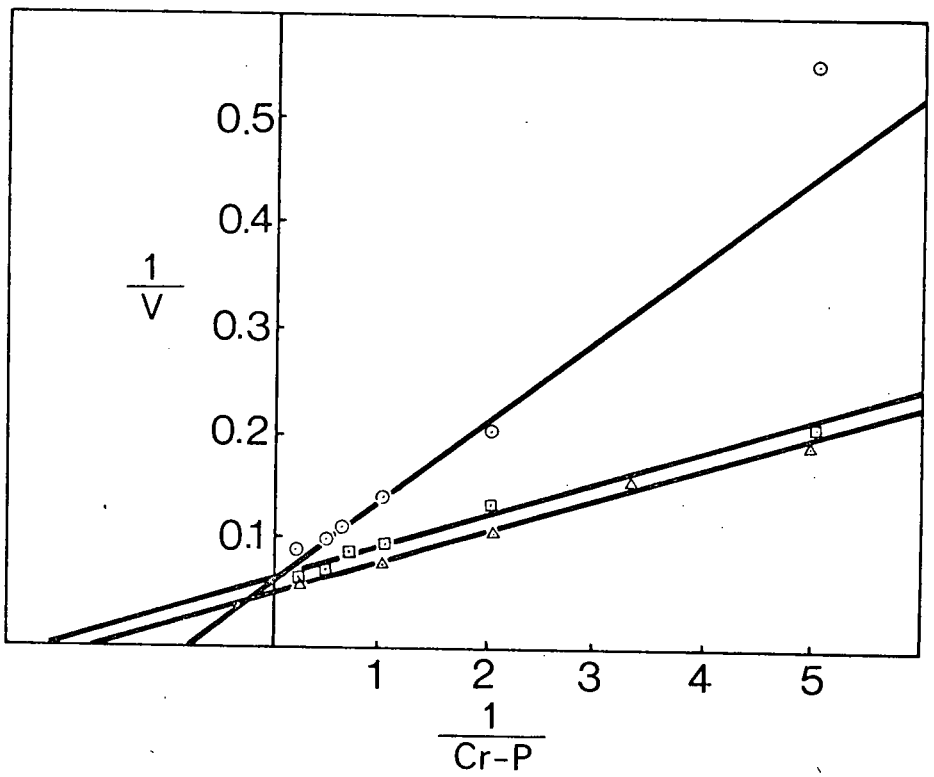
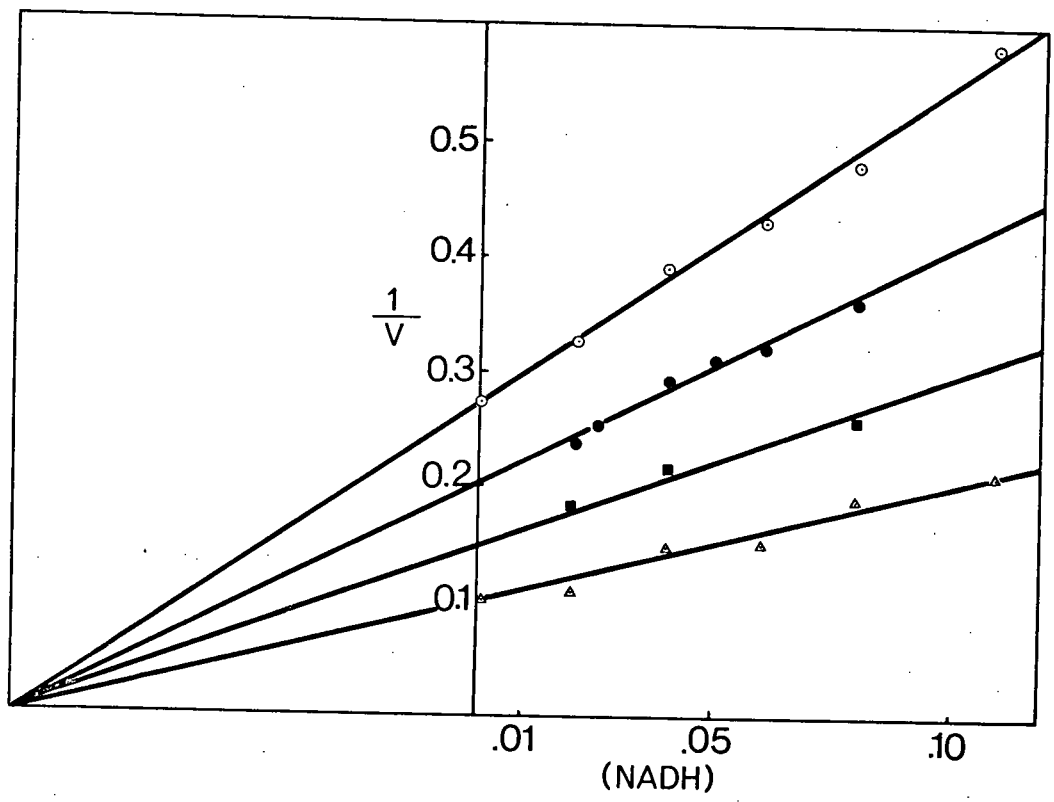

0.5
0.4
1/V
0.3
0.2
0.1
1
2
3
4
5
1/Cr-P

Figure 4. Dixon plot of NADH inhibition of creatine-P and ADP saturation kinetics of the mitochondrial enzyme. Reaction concentrations are: 20 mM imidazole buffer, pH 6.7, 5 mM $Mg(Acetate)_2$, 0.2 mM NADP, 4 mM glucose, excess dialyzed Sigma HK and G6PDH, varying concentrations of NADH and substrates at the concentrations below: 0, Δ, saturating ADP (0.2 mM) plus 1 mM and 5 mM creatine-P respectively; ⊗, ■, saturating creatine-P (5 mM) plus 0.05 mM and 0.07 mM ADP respectively.


0.5
0.4
1/V
0.3
0.2
0.1
.01
.05
.10
(NADH)

2.5 mM. The levels of creatine in mammalian tissue is between 20 and 30 mM (60) and so the reported K_m for creatine is close to physiological concentrations. Since the turtle heart has levels of creatine-P approaching 4 mM (4) the cytosol enzyme can be considered irreversible under physiological conditions.

Other Effectors

A series of metabolites were checked for effects at K_m values of substrates for each enzyme and the following were seen to have no effect: 5 mM alanine, 5 mM aspartate, 2 mM fructose diphosphate, 3 mM P-enolpyruvate, 3 mM 3-phosphoglyceric acid, 0.2 mM oxaloacetic acid, 4 mM glutamate, 3 mM pyruvate, 0.8 mM NAD, 3 mM malate, 0.2 mM GTP, 50 mM alpha-glycerophosphate, 5 mM succinate, 1 mM AMP, 50 mM lactate, 0.1 mM acetylCoA. Mg_2Citrate is a non-competitive inhibitor with a K_i value of 17 mM, much higher than *in vivo* concentrations (118). K^+ inhibits V_{max} activity about 20% at 100 mM.

DISCUSSION

Regulatory enzymes typically display two characteristics in common: they are under tight (positive and/or negative) metabolite regulation and physiologically behave as if they are irreversible (131). In no previous study has creatine kinase been shown to display these characteristics. In the turtle heart, however, we had two convincing reasons for expecting the cytosolic isozyme to behave as a regulatory site. In the first place, one of the substrates for the reaction, creatine phosphate, has a profound inhibitory effect upon turtle heart PFK, a key control site in glycolysis (103). Hence, control of creatine phosphate concentrations (by control of creatine kinase activity) would appear to be fundamental to glycolytic control. And secondly, other workers have shown empirically that during the early part of

hypoxic work the levels of creatine phosphate in turtle heart drop dramatically while the levels of ATP rise slightly, presumably due to creatine kinase facilitation (4). Hence, our study of turtle heart creatine kinase basically became a search of its regulatory properties.

In other organisms, two mechanisms (changing H^+ and ATP concentrations) have been proposed for creatine kinase control in muscle. As we have pointed out above, neither a dropping pH nor dropping ATP concentrations can account for creatine kinase activation in the turtle heart for neither "signal" appears to be large enough to bring about significant effects on the enzyme. That is not the case for NADH, however. Lai and Miller (130) have shown that when the oxygen supply to various tissues in the turtle becomes limiting, several oxidation-reduction (redox) systems in the cells become markedly reduced and in fact continue to become more and more reduced through the duration of the dive. In turtle heart, therefore, it is evident that NADH levels rise shortly after the outset of the dive, and these would serve to increase the affinity of creatine kinase for creatine phosphate, leading (1) to an activation of the enzyme, (2) to a drop in creatine phosphate concentrations, and subsequently, (3) to a deinhibition of PFK, and a consequent activation of glycolysis at a time when it is in fact required. Such a "one on-signal" control on creatine kinase would appear to be adequate to closely integrate the activity of the cytosol enzyme with the cell's glycolytic requirements, for the specificity of the NADH effect is high and the NADH concentrations required for full activation are well within expected physiological ranges (118). In contrast, 10-fold higher concentrations of NAD have no effect on enzyme catalytic rates and do not reverse the NAD activation.

Similarly, NADP and NADPH do not affect the cytosol creatine kinase isozyme.

Physiologically, creatine kinase in the cytosol is thought to function in the direction of ATP production; hence, its seeming irreversibility is not difficult to understand. It is important to emphasize, however, that the K_{eq} for the reaction is the same irrespective of the isozymic catalyst present, and that the apparent irreversibility stems from kinetic properties of the enzyme. The extremely low affinity of the cytosol enzyme for creatine and ATP kinetically precludes any significant reverse function of the enzyme under physiological conditions. Moreover, catalytic function in the forward direction is also strongly favored by the high affinities shown for creatine phosphate and ADP; in the absence of NADH, the turtle heart cytosolic isozyme displays a 3-fold greater affinity for both of these substrates than does the rat heart homologue, and this difference between the two enzymes is increased to nearly 9-fold in the presence of 0.1 mM NADH. Thus, an enzyme which on thermodynamic grounds is fully reversible in effect becomes a unidirectional catalyst on kinetic grounds. Other examples of such enzymes are known in the literature, a particularly well worked out system being described by Atkinson (132). In this property, the turtle cytosolic isozyme of creatine kinase differs fundamentally from its mammalian homologue (124). However, such behavior is a hallmark of many regulatory enzymes and would appear requisite for a creatine kinase isozyme whose activity *in vivo* must be governed by mechanisms other than mass-action effects.

In contrast to the cytosolic isozyme, the mitochondrial form of creatine kinase is thought to buffer the respiration rate of heart mitochondria by immediate production of creatine phosphate from ATP being formed by oxidative phosphorylation (127). That is, this isozyme is thought to function in the

backwards reaction in the cell. Interestingly, the kinetic constants for the turtle heart mitochondrial isozyme are between 3- and 6-fold higher than those for the rat heart analogue. The K_m found for creatine is 19 mM whereas the level of this metabolite (measured as creatine-P) in the turtle heart is only about 4 mM compared to 10 mM for the rat heart mitochondria (127). This extremely high value for the turtle enzyme could reflect the fact that in vivo mitochondrial creatine kinase is thought to be membrane bound and disruption of the system could affect the kinetic parameters (133).

Finally, it should be noted that although the electrophoretic patterns in turtle muscle and heart found here seem similar to the patterns seen for other animals (119), turtle creatine kinase does not co-purify with any other creatine kinase so far studied. Standard methods for the purification of the muscle enzyme include one or more of the following: pH 9.0 plus ammonium ion (119), ethanol precipitation (124), ethanol precipitation plus heat (123), and acetone fractionation (121). None of the above methods individually or in combination allow purification of the enzyme.

CHAPTER VI:

Summating Remarks

These studies on turtle heart phosphofructokinase, pyruvate kinase, and creatine kinase have suggested a number of conclusions concerning both the metabolic control of the enzymes and the underlying possible enzyme adaptations to fluctuations in oxygen availability during aero- and anaerobiosis. To conclude this study, a number of these will be discussed as well as probable solutions (based on our data or on the existing literature) to outstanding problems such as (1) redox balance in the terminal stages of diving, (2) utilization of substrate sources in diving, and (3) alternate end products produced in vertebrate divers . A metabolic map for probable control of anaerobic intermediary metabolism in vertebrate "facultative" anaerobes is developed.

The Anoxic Diving Turtle

In the Introduction it was pointed out that vertebrate skeletal muscle was already well adapted for anaerobic function compared to the liver which does not depend critically on glycolysis. In noting how Nature "tunes up" glycolytic machinery for efficient function we met the requirements necessary and the solutions that glycolytic tissues elaborated. Vertebrate white (skeletal) muscle is a good "anaerobic" tissue by virtue of its increased capacity for glycolytic generation of ATP. For my work I chose a normally aerobic tissue in a very "anaerobic animal" in the hopes that metabolic organization and control in the turtle heart is comparable to that described for skeletal muscle, possibly with even further exaggerations of a common theme.

Current information already confirms our observation that in compensating for temporary unavailability of O_2, a vertebrate activates anaerobic ATP yielding capacities by (i) increasing glycolytic potential: a higher titre

of both glycogen and glycolytic enzymes, and (ii) by modifying regulatory properties of key enzymes to keep that high potential under tight control: glycogen phsophorylase and lactate dehydrogenase.

This study deals with the control of carbon flux through anaerobic glycolysis. At the outset of a long dive, Pseudemys scripta exhibits normal aerobic metabolism: in the heart fat is degraded via the β-oxidation spiral to acetyl CoA, which feeds into the Krebs cycle, and NADH, which goes to the electron transport chain. ATP is produced by oxidative phosphorylation with O_2 as the terminal electron acceptor. Glycolytic flux is at a minimum due to inhibition of PFK by creatine phosphate (the storage form of energy) and to a lesser extent, citrate, which is a metabolite of the Krebs cycle. Since Pseudemys is unable to extract oxygen from water, as its body oxygen stores are depleted cellular oxidative metabolism slows.

As O_2 availability drops NADH levels begin to rise (intracellular redox system becomes more reduced) (4). This rise in NADH levels affects creatine kinase, the enzyme that metabolizes creatine phosphate. Very low NADH concentrations (0.04 mM) activate the reaction in the creatine phosphate hydrolyzing direction by lowering the K_m for creatine phosphate. This is the first step in glycolytic activation and the drop in creatine phosphate levels have a very specific effect on PFK, the key control enzyme of glycolysis.

The integrated function of the key glycolytic enzymes is so efficient that transient changes in ATP/ADP ratios are not detectable during aerobic-anaerobic transitions (35). Whereas a drop in ATP concentration is an important means for deinhibiting PFK and thus for "turning on" glycolysis in the rat heart during aerobic-anaerobic transition, it is not a useful signal in the heart of a diving turtle. The most unique change found in PFK from

the turtle heart is the enzyme's differential sensitivity to ATP and creatine phosphate. Compared to other PFKs the turtle heart enzyme appears to be surprisingly insensitive to ATP. It is about 200-fold less sensitive than sheep heart enzyme and about 1/3 as sensitive as is the rat muscle enzyme. There appears to be a gradation of PFK sensitivity to ATP depending on the glycolytic potential - the less sensitive the enzyme the more "glycolytic" the tissue - with turtle heart appearing at one end of the spectrum; more "poised" for glycolysis than white skeletal muscle. A similar spectrum exists for creatine phosphate effects: here the more glycolytic the tissue, the greater the sensitivity. Mammalian heart PFK is refractory to creatine phosphate, skeletal muscle enzyme somewhat inhibited, and turtle heart enzyme the most sensitive. The drop in creatine phosphate levels, then, serves to deinhibit PFK and allows increased glycolytic flux.

This deinhibition, moreover, is greatly potentiated by FDP, one of the products of the PFK reaction. FDP is known to momentarily accumulate during glycolytic activation and leads to a further PFK activation and thus to an autocatalytic increase in the rate of its own production.

The PFK of turtle heart has a higher affinity for F6P than other PFKs and shows hyperbolic instead of the normal sigmoidal saturation kinetics even at pH 6.9. This serves to "poise" it in the glycolytic direction even more but makes it harder to control since when it is deinhibited it is always <u>on</u> fully at physiological concentrations of substrate. So this enzyme is even more of an "on-off" system than a normal skeletal muscle PFK. It also has a much broader pH optimum curve and so has a higher percent activity at physiological pHs.

FDP control of glycolysis in the diving turtle is particularly pivotal, because, in addition to the above effects on PFK, it plays a key role in

integrating PFK activity with that of PK, the next major control site in the glycolytic pathway. In most mammals studied, the major integration mechanism merely involves adenylate coupling; that is, ADP, the product of the PFK reaction, is a substrate of PK, and this in itself serves to automatically coordinate the activities of these two enzymes. In lower vertebrates (fishes, reptiles), PK is an allosteric enzyme under close metabolite regulation and FDP, the other product of the PFK reaction, serves as a potent feed forward activator of muscle PK, assuring nearly simultaneous activation of both enzymes.

Table 1 is included to outline the metabolite regulation of the turtle heart PK.

TABLE 1

Summary of effectors of pyruvate kinase from turtle heart (20) and mammalian muscle (58)

Compound	Turtle	Mammal (rat)
ATP	inhibits	-
Alanine	inhibits	-
Phenylalanine	inhibits	-
Citrate	inhibits	
FDP	deinhibits	-
Creatine-P	-	inhibits

FDP control of PK is of two forms: firstly, it directly activates the enzyme by greatly increasing enzyme-substrate affinity, and secondly, 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 to account for an exponential rate of change of PK activity during aerobic-anaerobic transition.

Through these impressive adjustments in the glycolytic machinery, the turtle can compensate for the temporary depletion of O_2 by a large increase in glycolytic production of ATP, and so can greatly extend the length of the dive as well as the amount of work that can be performed during the dive. But even in the diving turtle, where these mechanisms reach their zenith, the animal must re-surface sooner or later and repay his O_2 debt.

This is where the story stands now - a series of inventive changes in the metabolic control machinery of a tissue to cope with a serious metabolic problem, oxygen depletion. So, underlying all the macroscopic levels of organization and their interrelationships is the biochemical machinery that allows all these processes to happen. And in any interesting biological problem - seemingly simple or obviously complex - especially one as basic as adaptation to an environmental stress, there is the subcellular world of enzyme adaptations to deal with. (Summary: Figure 1)

What Limits Survival in Turtle Diving

There are at least three possibilities as limiting factors: (1) low energetic efficiency of glycolysis, (2) dependence on carbohydrate as the sole energy source, and (3) increase in tissue acidity due to the production of lactic acid. Clark and Miller (4) suggest that the low energetic efficiency of glycolysis is the most important of these factors in the fresh-water turtle, since the concentrations of ATP and creatine phosphate in brain, heart and liver had declined after 3 hours of anaerobiosis, at which time the blood glucose concentration was elevated above normal, and intracellular acidity was only moderately increased.

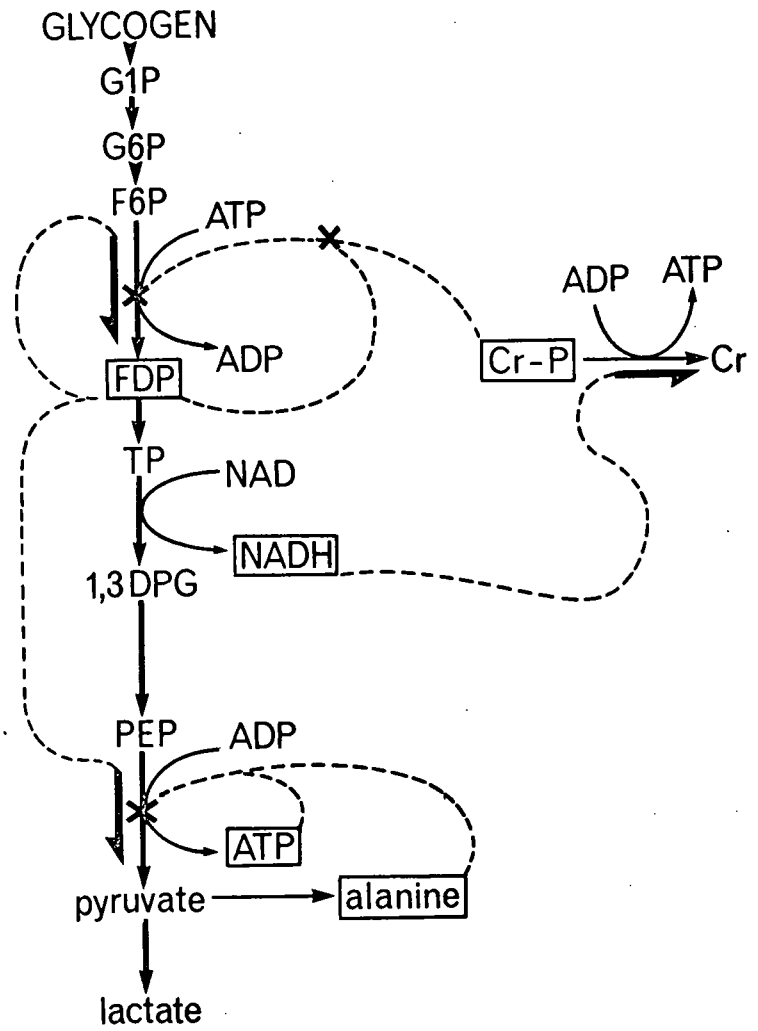


Figure 1. Summary of major glycolytic controls operative in turtle heart.

Blood glucose concentrations of 80 and 120 mg %, respectively, were seen in two turtles exposed to nitrogen to the point of death. Since the normal blood glucose concentration in the turtle is only about 60 mg %, the presumption is that the substrate for glycolysis was not limiting even at the time of death. It is possible, however, that a sustained hyperglycemia might, by mass action effect, maintain a higher rate of glycolysis in the later phase of anaerobiosis. The early depletion of cardiac glycogen and the low and relatively unchanging concentration of brain glycogen, during anaerobiosis, emphasize the dependence of these organs on glucose derived from liver glycogenolysis. The very high levels of blood glucose reached during prolonged oxygen deprivation reflect the exuberance of this reaction in the turtle and suggest that it may be of adaptive value.

The third of the suggested limiting factors in anaerobic survival of the turtle is that of an intolerable increase in intracellular acidity. The drop in intracellular pH in turtle heart over a 12 hour period of anoxia was only 0.3 of a pH unit, certainly not enough to cause tissue damage but it may nevertheless be of some importance. It is well established that the rate of glycolysis is decreased by acidosis, and this may be the cause of the apparent decreased rate of glycolysis seen after 3-6 hours of anaerobiosis.

Diving and the Maintenance of Redox Balance

Redox balance during early phases of anaerobic glycolysis in muscle is maintained by a functional 1:1 activity ratio between glyceraldehyde-3-P dehydrogenase (GAPDH) and lactate dehydrogenase (LDH). Both enzymes occur in higher titre in vertebrate muscle than in other tissues (23), and occur in about a 1:1 ratio. Towards the end of a dive, lactate concentrations are very high, so high that through mass action effects further conversion of

pyruvate becomes retarded and pyruvate either is transaminated to alanine or accumulates (134). This situation leaves glycolysis out of redox balance and if it is to continue the GAPDH oxidation step must have some other source of NAD. That source could be the α-glycerophosphate dehydrogenase (α-GPDH) reaction, in which case, α-glycerophosphate accumulates as an additional anaerobic end product (135). α-GPDH levels in the muscle of a mammalian diver (the porpoise) are two fold higher than levels of LDH from the same tissue (27). Presumably, some controlling mechanism (perhaps one as simple as just rising levels of NADH) is available so as to "turn on" α-GPDH at the correct time.

Another possibility in maintenance of redox balance in glycolysis towards the end of diving is shown in Figure 2 . Owen and Hochachka (136) have proposed that in porpoise muscle redox balance is maintained during extreme anoxia by a functionally 1:1 coupling between alanine and aspartate aminotransferases. For this purpose, alanine aminotransferase occurs in porpoise muscle at concentrations up to 15-fold higher than in terrestrial mammals (136), and so effectively competes for pyruvate; as a result, alanine accumulates as an additional anaerobic end product in the porpoise (134). They suggest that it is coupled to aspartate aminotransferase, which also occurs in unusually high titre, in order to account for the correlation between alanine accumulation and aspartate depletion (134). This arrangement sets the stage for MDH catalyzed reduction of OXA (the alternate product of the aspartate aminotransferase step) to malate. It assures a stoichiometric relationship between pyruvate accumulation and malate accumulation. That is, it assures a mechanism that in effect "turns on" MDH to the degree necessary for balancing redox in the glycolytic path,

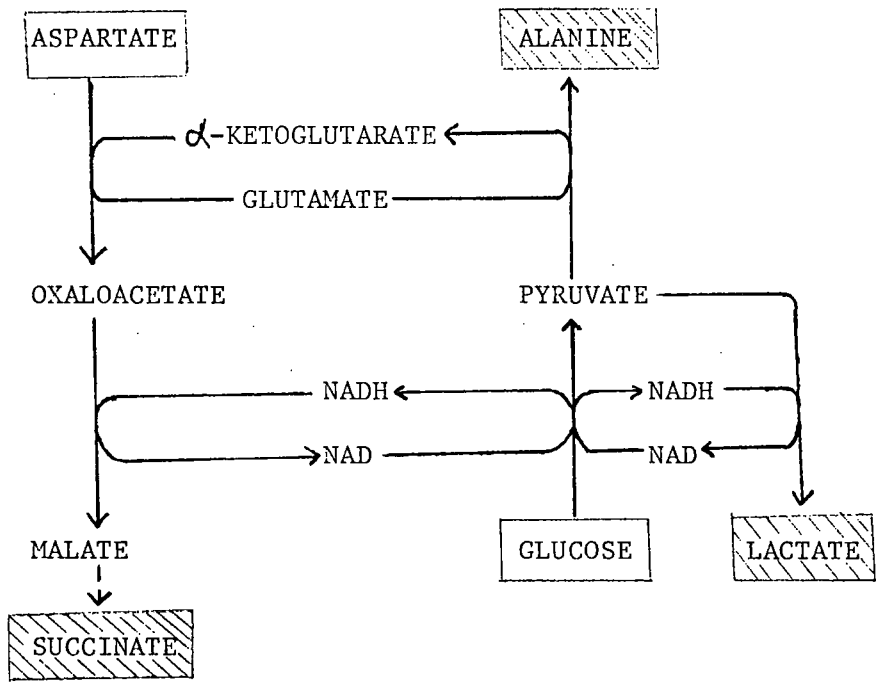


Figure 2. Coupled transaminases to balance redox in the diving porpoise.

because the signal for turning on this transaminase couple (pyruvate accumulation) is also the measure of the degree to which the system is out of redox balance. Although this mechanism may serve to help maintain redox balance in mammalian divers it is seemingly an unnecessary frill for the diving turtle who is unperturbed by large redox imbalances (129). Perhaps another "spectrum" argument can be used here in the tolerance of animals to rising NADH levels. In a non-diver like the rat redox is kept very constant and in anoxic stress there are very efficient methods of reoxidizing the NADH formed. Perhaps the diving animals have adapted to cope with the high levels of NADH produced in their muscles like they have elaborated mechanisms to deal with large amounts of lactate (137). The redox balance problem could be much less critical to the mammalian diver than to other animals. The aforementioned redox balancing mechanisms could be important in other tissues such as the brain, however (D. Jones, personal communication).

Substrate Utilization in Diving

a) Lipid "sparing" in anaerobic glycolysis:

The problem of inhibiting lipid metabolism during anaerobiosis is not unique to diving mammals. In better studied species, fatty acid oxidation is dampened during anaerobic glycolysis. When lactate levels increase, they serve to inhibit the mobilization of triglyceride by inhibiting lipase, the first step in fat catabolism. In addition, the mobilization of adipose triglyceride is under hormonal control and this is thought to contribute to the required integration between triglyceride and glycogen metabolism. One could speculate that aerobic triglyceride catabolism in red and intermediate type fibers of the marine mammals (138) is under this same hormonal control by direct effects on the high lipase content of

of these muscles (138). During prolonged diving, when blood flow to the muscle is shut off, such hormonal signals also would be automatically "turned off" thus leading to a drop in the ratio of active/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 favoured.

b) Activation of aerobic metabolism:

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 as it is activating fat metabolism. When oxgyen returns and fatty acid oxidation is initiated, fluctuations in various Krebs cycle intermediates occur. Citrate concentrations increase greatly and this metabolite serves to feedback inhibit PFK, thus effectively blocking glycolysis and sparing carbohydrate stores (118). This mechanism is used in all tissues of the vertebrate, but as already pointed out, muscle PFK is unusually citrate sensitive (about 10 times more so than the liver homolog), and thus in muscle this control interaction is perhaps most effective. As far as it is known, the same control mechanism at this locus in glycolysis also operates in tissues of diving vertebrates (103), but whereas this seems to be a sufficient mechanism in muscles of typical terrestrial mammals, it is not the method elaborated to "turn off" glycolysis during aerobic periods in diving vertebrates. In vertebrate divers, pyruvate kinase is also an allosteric enzyme, is highly sensitive to citrate, and thus would supply an additional means for blocking glycolysis during periods of fat catabolism (20). A third and most important mechanism for blocking glycolysis at this time involves alanine inhibition of pyruvate kinase.

In muscle during anaerobic/aerobic transition, activation of the β-oxidation spiral leads to a momentary piling up of acetylCoA as OXA reserves for citrate synthesis are inadequate (139). In a diving animal such a limitation could be crippling. Thus, the diving habit leads to another important metabolic requirement in muscle: an efficient source of OXA that can be "turned on" during fatty acid oxidation and Krebs cycle activation. The source of OXA is aspartate via the aspartate aminotransferase step. As already noted in marine mammals, such as the porpoise, the activity of this enzyme in muscle is higher than in terrestrial species (136), and its catalytic properties are such as to favour OXA production in the mitochondria during anaerobic/aerobic transition. Glutamate, produced in the reaction, in turn transaminates with pyruvate to regenerate α-KGA, a process leading to alanine accumulation. The total amount of alanine accumulated under such conditions is equal to the summed increase in concentration of all Krebs cycle intermediates (139).

Alanine then is perhaps the "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 PK in diving vertebrates (20,27), for the greater the degree of Krebs cycle activation, the greater the degree to which PK is alanine-blocked and carbohydrate reserves are "spared". Alanine inhibition is 200-fold more effective for turtle heart PK (20), and 10-fold more effective for porpoise muscle PK (27) than it is for the mammalian muscle enzyme (see Figure 1).

Alternate End Products, or Are Non-Glycolytic Anaerobic Mechanisms Functional in Vertebrates?

Intertidal bivalves and other invertebrate facultative anaerobes utilize "extra-glycolytic" substrate level phosphorylations to produce ATP. These

reactions involve the simultaneous mobilization of carbohydrate and amino acids and produce largely succinic acid (8).

Several groups have looked at the quantitative significance of amino acid fermentation in diving vertebrates. Studies have been done on mammals, such as the rat (140,141) and the guinea pig (142), in the diving turtle (67) as well as in another reptile, Testudo hermanni (143). In many instances of anoxic or hypoxic stress, succinate has been shown to accumulate, but peak concentrations are 1-2 orders of magnitude lower than are those for lactate. Studies have been done on blood concentrations of succinate in some diving animals (134). Problems arise in these studies because succinate is produced in the mitochondria and as soon as the dive finishes it will be quickly oxidized. However, since diving is accompanied by extensive peripheral vasoconstriction, muscle metabolism becomes a closed system. If metabolites such as succinate are produced during the dive, they accumulate and during initial recovery, when the blood circulation to the muscle is reopened, there is a good chance that some of the succinate will be "washed out", even if most of it is oxidized. Blood succinate concentrations following diving are about 1/100 the concentrations of lactate. If we assume that the lactate:succinate concentration ratio in the blood accurately reflects the tissue ratio, then amino acid fermentation produces about 1/100 as much ATP as does glycolysis. This is probably a low estimate for the contribution of these pathways, but does reflect the fact that they are minor compared to the production of lactate.

Amino acid fermentation is probably of peripheral importance to energy metabolism in most vertebrate species because: 1) of the small free amino acid pools present in vertebrate muscles. The invertebrates that employ this

method have large amino acid pools for osmoregulation. To be a useful energy source there must be a high concentration of substrate. And 2) the animal must eventually repay its oxygen debt following anoxic excursions. The conversion of PEP to succinate by the oyster pathway yields 2 moles of ATP/mole of succinate formed, while the conversion of PEP to succinate by the aerobic (Krebs cycle) route yields 11 moles of ATP/mole of succinate formed. That is, if the animal can afford to "wait out" the anoxia by storing pyruvate (as lactate) and then catabolizing that pyruvate to succinate by aerobic routes, it gains over 5 times the amount of useful energy than it would if it converted pyruvate to succinate during actual anoxia. And since these animals must return to air and have to live with the end product that they produce, lactate is the most suitable one.

Several studies with various invertebrates (144), turtles (145), and other vertebrates (146,147) have found increased biosynthesis of fats in anoxia. Although the synthesis of fat requires large amounts of ATP, which would be at premium in anoxic stress, this mechanism utilizing NADPH or NADH may act as an electron acceptor for some of the NADH generated in anaerobic glycolysis (144). Arillo and de Giuli (145) found fatty acid synthesis only in severely stressed turtles, pointing up that due to the massive energy requirements, this is a last ditch mechanism.

Other possible non-glycolytic end products already have been mentioned: glycerol (135) formed from the buildup of alpha-glycerophosphate seen in anoxia (135), and thought to be involved in redox balance; and alanine, the product of the two transaminases (136) postulated to be involved in redox balance in terminal stages of diving stress.

CHAPTER VII:

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Appendix

APPENDIX

Belkin, 1963 (62) reports a great diversity in submergence times for various turtle families. There were several species within the subfamily Emydinae (family Tsetudinidae) that might have been used in this study - especially Chrysemys (painted turtle) and Pseudemys (red-eared pond slider).

On repeated observations Pseudemys seemed the most "at home" under water. When put in tanks without access to air, Chrysemys displayed much less of an ability to "dive" at 15-18°C. To test these observations against the theory (6,22) that increased diving time correlates with increased levels of the control enzymes of glycolysis, PFK and PK, activities of these enzymes were measured in the brain, heart, red muscle and white muscle of each of the turtles.

Each tissue was homogenized (25% homogenate) in 50 mM Tris-HCl pH 7.5, 1.5 mM dithiothreitol, 2 mM $MgSO_4$, and 1 mM EDTA. For PFK 10 mM F6P was also added to the medium. Assays were performed on the high speed supernatant and the enzymes were assayed as in Materials and Methods. Enzyme profiles are shown in Figures 1 and 2 . Activities are expressed as micromoles of substrate converted per minute per milligram of protein.

Pyruvate kinase levels in the heart (the tissue that I chose to study) are higher in Pseudemys as are the levels in white muscle. However, brain and red skeletal muscle levels are the same for both turtles. We have since found that PK levels are a poor choice to compare divers and non-divers. The porpoise, an excellent mammalian diver, has lower absolute specific activities of PK than the non-diving rat but has a different type of PK that allows for greater glycolytic flux when it is needed (27).

PFK levels in each tissue tested were higher in Pseudemys with the

largest difference seen in the heart. This would indicate that the better diver has geared protein synthesis to its need for increased glycolytic flux in diving. Although increased levels are not conclusive in this regard, it is general that not only is more enzyme produced in response to environmental stress but there is also a different, more "adaptive" variant produced (11).

The trend then is that the best diver has the highest level of enzymes with PFK being the best indicator. PFK is the known major control point in turtle heart glycolysis (35).

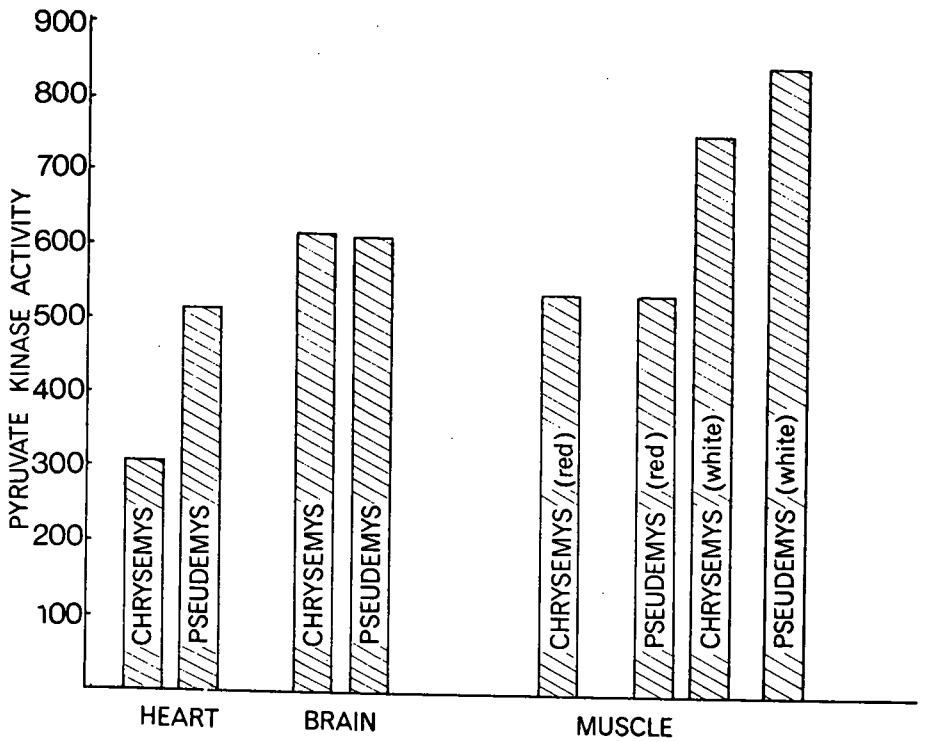

Figure 1. Levels of pyruvate kinase present in selected tissues of Pseudemys and Chrysemys .

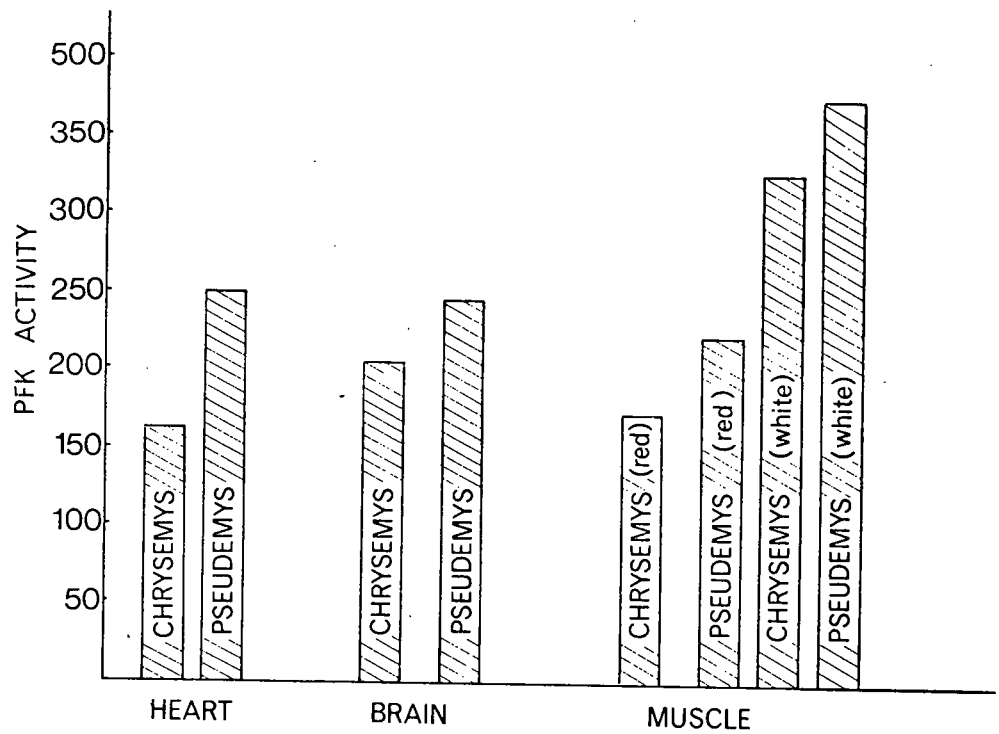

Figure 2. Levels of phosphofructokinase present in selected tissues of Pseudemys and Chrysemys.

Appendix

Abbreviations used:

PFK - phosphofructokinase

PK - pyruvate kinase

CK - creatine kinase

LDH - lactate dehydrogenase

G3PDH - glyceraldehyde-3-phosphate dehydrogenase

MDH - malate dehydrogenase

G6PDH - glucose-6-phosphate dehydrogenase

HK - hexokinase

ATP, ADP, AMP - adenosine tri-, di- and monophosphate

NAD, NADH - oxidized and reduced forms of nicotinamide, adenine dinucleotide, respectively

NADP, NADPH - oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate, respectively

creatine-P, CrP - creatine phosphate

F6P - fructose-6-phosphate

fructose-1,6-P_2, FDP - fructose diphosphate

cAMP - cyclic AMP; adenosine 3':5'-cyclic monophosphatic acid

2PGA, 3PGA - 2-, 3-phosphoglyceric acid

FFA - free fatty acids

P-enolpyruvate, PEP - phosphoenolpyruvate

G6P - glucose-6-phosphate

Nitro BT - nitro blue tetrazolium

PMS - phenazine methosulfate

OXA - oxaloacetate