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SMULLIN, DAVID HYAM
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UNIVERSITY OF ALASKA, M.S., 1980

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THE REGULATION OF ENZYME ACTIVITY IN A HIBERNATOR: 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM LIVER OF THE ARCTIC GROUND SQUIRREL

A

THESIS

Presented to the Faculty of the
University of Alaska in partial fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE

By

David Hyam Smullin, B.A.
Fairbanks, Alaska

May, 1980

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THE REGULATION OF ENZYME ACTIVITY IN A HIBERNATOR:
6-PHOSPHOGLUCONATE DEHYDROGENASE FROM LIVER OF THE
ARCTIC GROUND SQUIRREL

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ABSTRACT

The properties of hepatic 6-phosphogluconate dehydrogenase (6PGD) from the Arctic ground squirrel were examined over the seasonal physiological temperature range of the animal.

While there are seasonal differences in 6PGD activity, the affinity of the enzyme for both of its substrates, as well as the isoelectric point remain unchanged over the year. The data suggest that seasonal control of this key enzyme of the hexose monophosphate shunt is chiefly through changes in enzyme concentration.

The metabolites adenosine diphosphate (ADP), adenosine monophosphate (AMP), Mg^{++} and fructose-1,6-bisphosphate (FBP) have no effect on enzyme activity, while AMP is mildly inhibitory (Ki=2.18 mM). NADPH is a potent inhibitor (Ki=0.02 mM), and may be a major regulator of 6PGD activity.

The literature on regulation of the hexose monophosphate shunt is reviewed, and a tentative model for regulation of the shunt in the liver of the Arctic ground squirrel, explaining the differences in hexose monophosphate dehydrogenases between hibernating and nonhibernating animals, is proposed.

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ACKNOWLEDGMENTS

I thank the following people:

Dr. Hans Behrisch for his sponsorship*, advice and encouragement;

Mr. Declan Troy for his help with the computer and statistical analysis;

Drs. Plaur Blair, John Bligh, Don Button, Dale Feist, Josephine Rauch and Steve MacLean for their advice, and/or criticisms of the manuscript;

My fellow graduate students for their thoughtful discussions and advice during the project, and criticisms of the manuscript;

My wife Terry Bonynge for her patience, encouragement and sponsorship.**

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*This research was partially funded by a grant from NIGMS No. 10402.

**The author was partially funded by grant No. 1 from the Terry Bonynge Foundation.

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> The phenomenon of hibernation which has attracted the interest of zoologists for more than 200 years, provides the first demonstration in comparative physiology of a striking adaptation to special environmental requirements, and at the same time emphasizes the contrasting homeostatic conditions in other mammals [Morrison 1960].

INTRODUCTION

The vast inter-connectedness of the many metabolic pathways in the anabolic and catabolic conversions of organic compounds required by living organisms can be a mind-boggling tangle to unravel. The biochemist attempting to elucidate the links and points of control involved in metabolism and body function is faced with a problem that at first glance seems almost insurmountable. Luckily, there are certain basic properties and universal substances involved in key roles in the control and integration of metabolic pathways. With an understanding of the roles of the various compounds acting as transporters of energy, and of the important factors inherent in enzymatic reactions acting as control points of metabolic pathways, the biochemist can begin to unravel this tangle. The elucidation of these pathways over the past 50 years has pointed out the simplicity and economy inherent in them, thus allowing for the beginning of an understanding of their integration and control.

The primary job of metabolism is to supply usable energy to the cell. Energy for the cell is derived from the energy

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inherent in the structures of organic molecules obtained from the surroundings. Energy and electrons derived from fuel molecules are transported from catabolic reactions to electron and energy-requiring anabolic reactions in the high-energy phosphate bonds of adenosine triphosphate (ATP), and the energy-rich electrons of reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Regulation of metabolic pathways is attributed to enzymes situated at key points in the pathways (usually at the beginning and branch-points) catalyzing physiologically irreversible reactions. Allosterism, the modulation of enzyme activity through the binding of a specific metabolite at a site other than the catalytic site, is a common feature of many regulatory enzymes. An important characteristic of allosteric enzymes is the sigmoidal substrate saturation curve, in contrast to the common rectangular hyperbola. The sigmoid curve implies that binding of one substrate molecule to the enzyme enhances the binding of subsequent substrate molecules to other substrate sites. The shape of these curves allows for large changes in enzyme activity caused by small changes in substrate concentrations, if substrate is physiologically present at concentrations approximating the Km (the substrate concentration required for an initial velocity of 1/2 maximal velocity) of the enzyme for substrate. Most modulators of regulatory enzymes act by affecting the affinity of the enzyme for its substrate,

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thus affecting the crucial relationship between Km and physiological concentrations of substrate.

Glycolysis

The glycolytic pathway was one of the first major pathways to be studied, and serves as a backbone for the rest of intermediary metabolism. It carries out the anaerobic degradation of the six-carbon sugar, D-glucose, to the three-carbon compound, pyruvate. In anaerobic organisms pyruvate is reduced to either lactate or ethanol, and the pathway acts as the major producer of high-energy ATP molecules. In most aerobic organisms this pathway has been retained and serves as a preparatory pathway for aerobic metabolism and production of ATP via the tricarboxylic acid cycle and electron transport system. It also serves aerobic organisms as an emergency source of energy during short periods of anoxia (Fig. 1).

This 11-step pathway is generally thought to be under the control of the three reactions catalyzed by the enzymes hexokinase (HK) (or glucokinase in liver), phosphofructokinase (PFK), and pyruvate kinase (PK), all of which catalyze irreversible rate-limiting reactions. This can be determined from examination of the large changes in their standard free energy ($-\Delta G°$) and mass action ratios. Regulation of the pathway stems from the concerted effect of all three.

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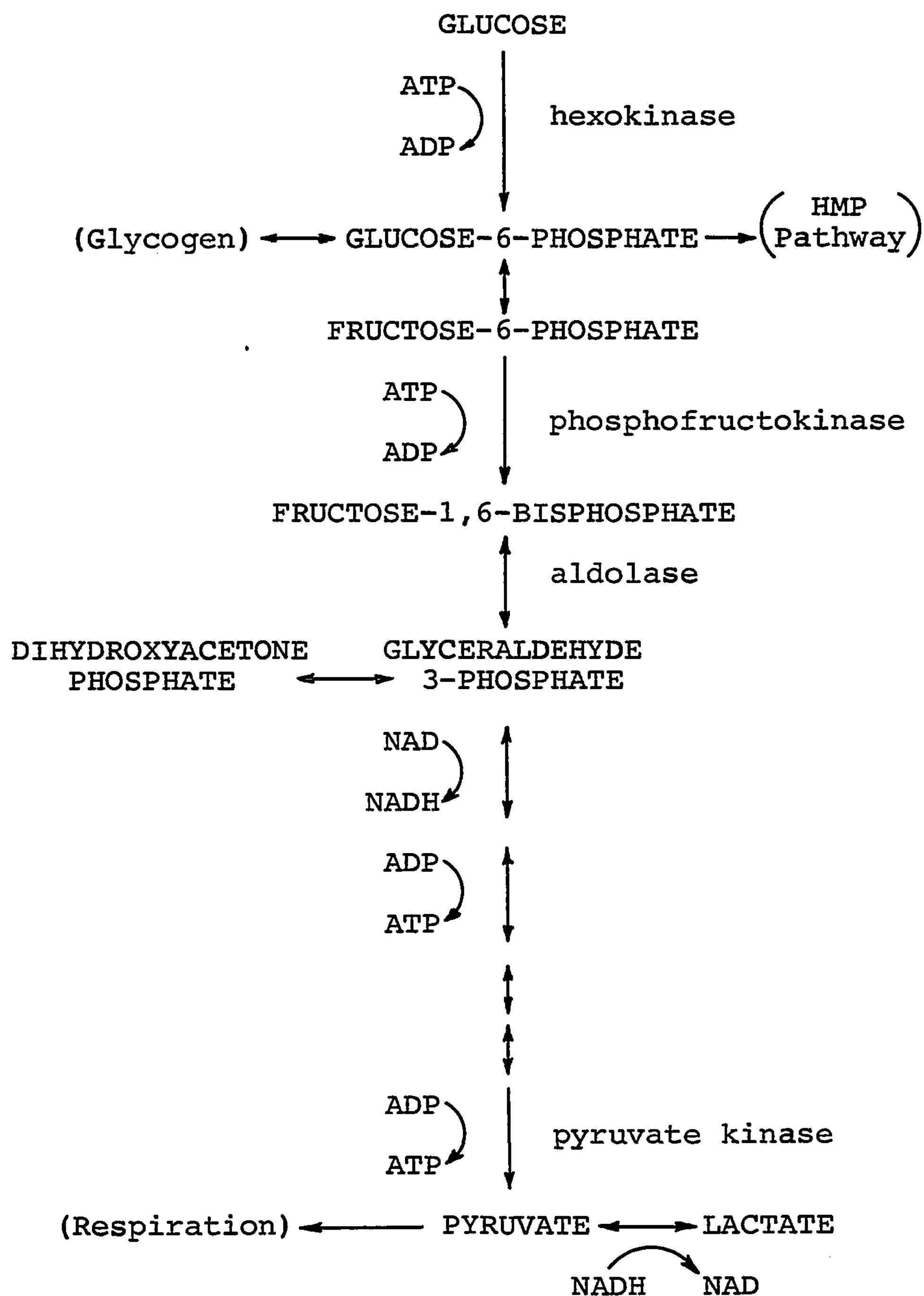

Fig. 1. Glycolysis.

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PFK is inhibited by high concentrations of ATP (a substrate of the enzyme, but a product of the overall pathway), citrate, and long chain fatty acids. It is activated by AMP and ADP, which are present in high concentrations when the product (ATP) is low. Thus PFK is poised to drive glycolysis when the energy charge is low (characterized by low concentrations of ATP and high concentrations of AMP+ADP), and to inhibit the pathway when its product and high-energy molecules are plentiful. In this study, inhibition by long chain fatty acids is important, as a major role of glycolysis in the liver is the biosynthesis of fatty acids.

Hexokinase is noncompetitively inhibited by its product, glucose-6-phosphate (G6P) (Newsholme and Start 1973), which builds up when PFK is inhibited. The noncompetitive inhibition is one of the cornerstones of the concerted effect of PFK/HK regulation. If G6P inhibition were competitive, an increased glucose concentration would activate the enzyme. This would increase fructose-6-phosphate (F6P) concentrations, which would override the inhibition of PFK by ATP, and thus there would be no control of the pathway. In the liver, where glucokinase replaces hexokinase as the predominant form of the enzyme, glycolysis is not the only important pathway for glucose degradation. In view of this, it is not surprising that glucokinase is not inhibited by G6P, and that increased blood glucose drives the reaction

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towards G6P formation under most conditions. This is an important factor in the activity of the hexose monophosphate pathway (HMP), which branches off from the glycolytic pathway at the level of G6P.

Although pyruvate kinase catalyzes a reaction that is far removed from equilibrium, and is inhibited by a number of products of glycolysis, while being activated by its substrate and fructose-1,6-bisphosphate (FBP), its role as an important regulator of glycolysis is questionable. Its position at the end of the pathway, and high activity in comparison to PFK, contribute to this uncertainty and suggest that inhibition of this enzyme would lead only to accumulation of glycolytic intermediates between FBP and phosphoenolpyruvate (PEP).

Hexose Monophosphate Pathway

The hexose monophosphate shunt branches off from the main glycolytic pathway at the level of G6P, and diverts some of the glucose in the cell away from the production of ATP by oxidation (Fig. 2). The principal role of this pathway in most cells is to generate reducing power in the cytosol in the form of NADPH, a cofactor required in the reductive reactions involved in the synthesis of fatty acids and cholesterol. This function is particularly important in liver and mammary gland, which actively synthesize these substances. Not surprisingly, the pathway is active in these

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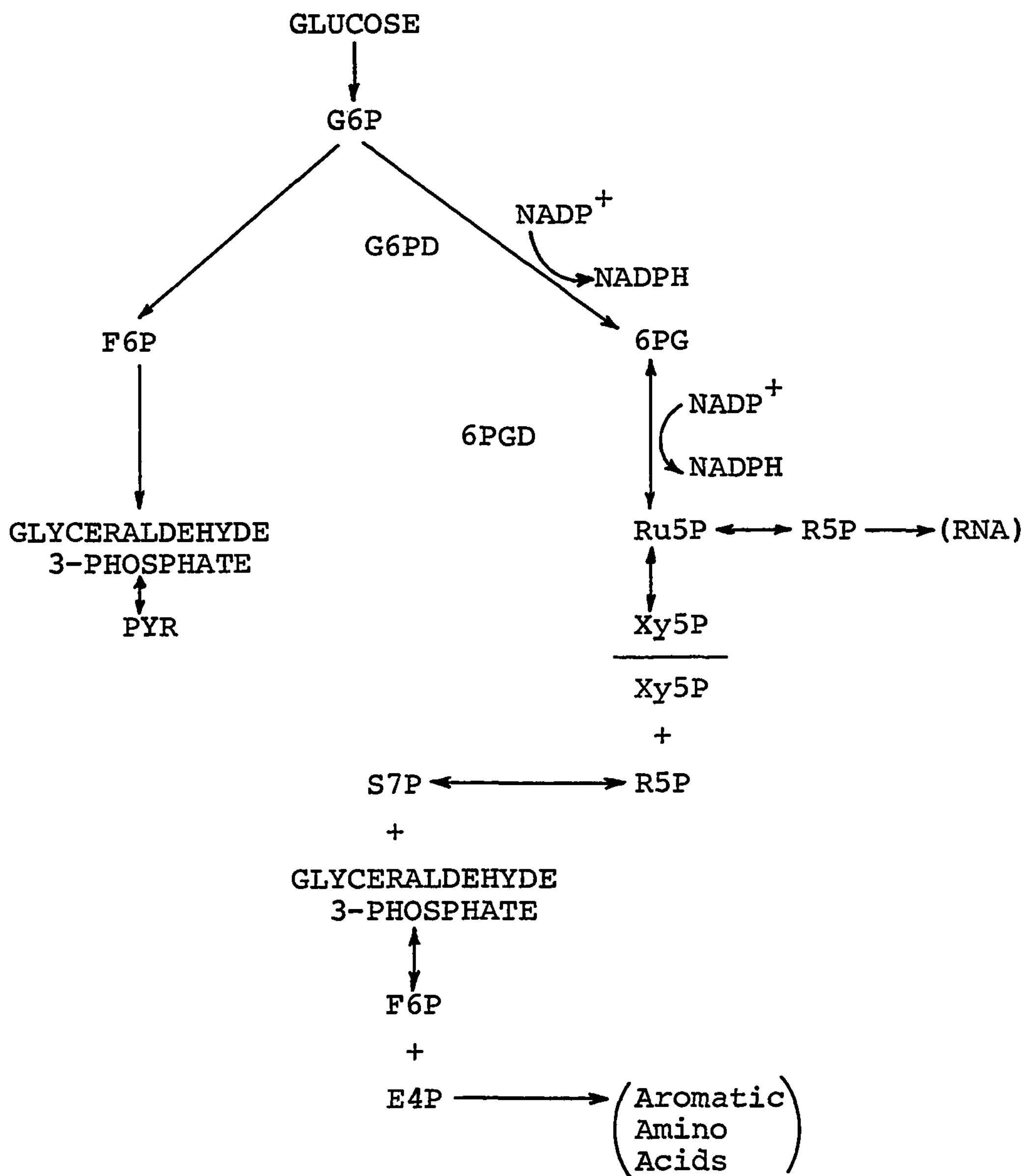

Fig. 2. Hexose monophosphate pathway.

Key:
PYR =pyruvate
Ru5P=ribulose 5-phosphate
R5P =ribose 5-phosphate
Xy5P-xylulose 5-phosphate
S7P =sedoheptulose 7-phosphate
E4P =erythrose 4-phosphate

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tissues and as much as 50% of all glucose undergoing degradation in the liver may enter it (Lehninger 1975), while in skeletal muscle the pathway is almost nonexistent. Another less important function of the pathway is the conversion of hexoses to pentose phosphates for the synthesis of nucleic acids. The final role of this pathway in animal cells is the complete oxidative degradation of pentoses by converting them to hexose intermediates of glycolysis. This function of the pathway has been suggested as a bypass of PFK control of glycolysis, since G6P is converted to pentose phosphates and then to the triose intermediates that are produced after the PFK reaction. This bypass could be active in the removal of high concentrations of glucose by the liver during the first two hours of refeeding after starvation (Newsholme and Start 1973).

The HMP link with fatty acid synthesis lies in the first three reaction steps in which the two dehydrogenases, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), catalyze reactions producing one molecule of NADPH each. These reactions are also the most probable control points of the pathway, with the linked reactions catalyzed by G6PD and lactonase being the only irreversible steps. Their placement at the beginning of the pathway, following a branch-point, suggests a role as the committing step. The modulation of both G6PD

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and 6PGD activities by products of the pathway and by glycolytic intermediates suggests that even though these enzymes may not be "regulatory" in the strict definition, they certainly play a role in the apportioning of G6P between glycolysis and the HMP.

Fatty Acid Synthesis

The formation of the fatty acids can be divided into two parts: the carboxylation of acetyl-CoA to form malonyl-CoA (catalyzed by acetyl-CoA carboxylase); and the series of condensation, dehydration and reduction reactions, starting from acetyl-CoA and malonyl-CoA. This series of six reactions is catalyzed by the fatty acid synthetase system, a complex of six enzymes and a nonenzymatic carrier protein (ACP) to which the growing fatty acid chain is attached.

The acetyl-CoA carboxylase reaction appears to be the regulatory, rate-limiting step in fatty acid synthesis. This enzyme catalyzes a nonequilibrium reaction with a maximal activity much lower than that of the fatty acid synthetase complex. It also appears to be an allosteric enzyme, made up of a number of inactive monomers that are shifted to the active polymer form only in the presence of citrate (see Lehninger 1975), and is inhibited by long chain acyl-CoA derivatives, a product of the pathway (Numa et al. 1964). The activation and inhibition by changes in

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concentrations of these compounds have not been shown at physiological concentrations, which casts some doubt on their roles in regulation of the enzyme's activity *in vivo* (Fang and Lowenstein 1967).

The two reduction reactions of the fatty acid synthetase complex are:

1) Acetoacetyl-S-ACP + NADPH + H ←——→ d-β-Hydroxybutyryl-S-ACP + $NADP^+$

2) Crotonyl-S-ACP + NADPH + H ←——→ Butyryl-S-ACP + $NADP^+$.

They are catalyzed by β-ketoacetyl-ACP reductase and enoyl-ACP reductase (NADPH) respectively, and have a specific requirement for NADPH as a cofactor. If the production of NADPH by the HMP and other reactions (catalyzed by malic dehydrogenase and isocitrate dehydrogenase) were limited, these steps could become rate-limiting in the lipogenic pathway (Siperstein and Fagan 1958). The actual extent of control of lipogenesis by the rate of NADPH production by the HMP is questionable, and Tepperman and Tepperman (1961) present convincing arguments for the regulation of the HMP dehydrogenases by lipogenesis at the initial stages of increasing lipogenesis in refed rats.

Glucose-6-Phosphate in the Liver

Most incoming nutrients pass directly from the intestine to the liver, which acts as a major sorting center of

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nutrients in the hierarchy of organ systems. In line with this role, the liver has great flexibility in terms of induction and repression of specific enzymes required to metabolize the varying mixture it receives.

The liver receives a large quantity of various monosaccharides, approximately 2/3 of which are phosphorylated and transformed to G6P. G6P in turn has a number of metabolic pathways that it can take in the liver (Fig. 3). Under a normal diet most of the G6P is converted to glycogen, fatty acids or blood glucose. Most of the ATP required by the liver is provided by the degradation of fatty acids and amino acids, and little G6P is completely oxidized in the liver. The HMP in the liver is one of the major producers of NADPH for fatty acid synthesis. According to Lehninger (1975), about 50% of all the glucose degraded in the liver enters the HMP, but Baquer et al. (1973) report a lower value of approximately 16%.

Control of the HMP

Regulation of metabolic pathways is usually attributed to enzymes having specific properties, as discussed earlier in this section. Modulation by these enzymes is carried out by changes in the activities of the enzymes, causing changes in flux through the pathway that compensate for, or relieve the effects of environmental change and stress. According to Behrisch (1973), there are at least three general methods

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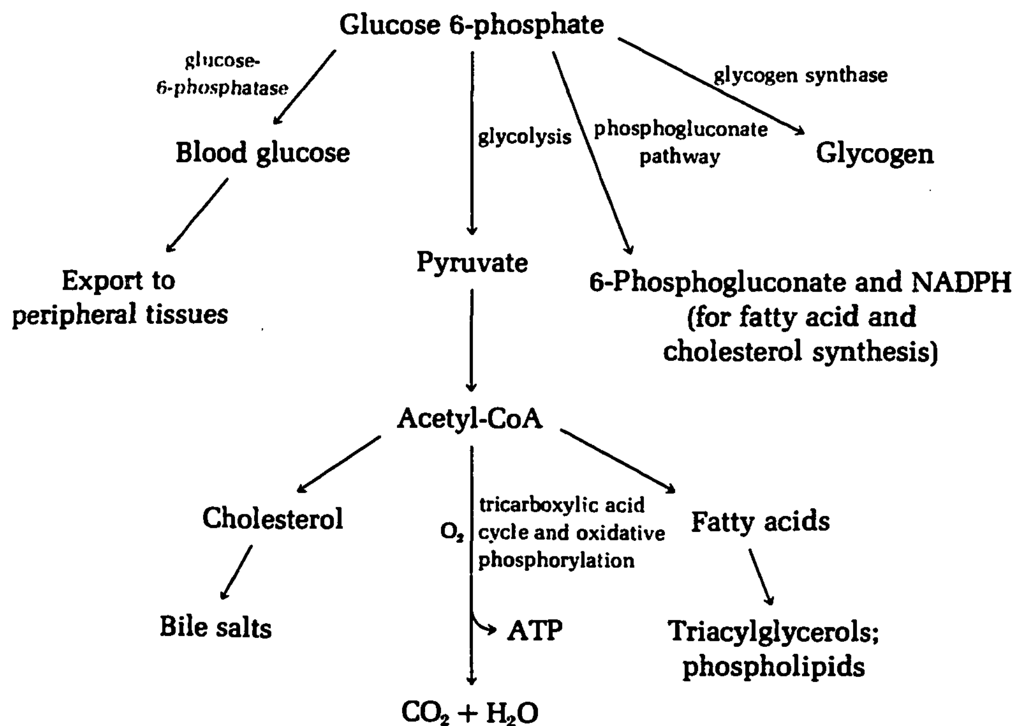

Fig. 3. Metabolic pathways of glucose-6-phosphate in the liver (Lehninger 1975).

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that organisms have of altering activities of enzymes in the cell: 1) altering enzyme concentrations by induction and repression of enzyme synthesis at the level of transcription and translation, and by changes in rates of degradation of active enzymes. Adaptations of this kind require a fair amount of time to produce maximal effects, and have been termed "coarse" control by Krebs and Eggleston (1974). 2) Production of enzyme variants or "isozymes" exhibiting differing kinetic properties; and 3) changing the "internal milieu" (i.e. concentrations of substrates, cofactors, or modulators of the reaction) of the cell. The latter two types of adaptation occur more or less instantaneously, usually by allosteric effects, and have been called "fine" control (Krebs and Eggleston 1974). Of course it must be realized that these types of adaptations are not mutually exclusive, and are more likely to work in concert to some degree.

In the past the first of these mechanisms has been invoked liberally to explain many instances in which a change in enzyme activity has been observed. Often this has been done with no additional information, but in the last 20 years a number of techniques have been developed which allow determinations of changes in enzyme level. The use of drugs that inhibit RNA and protein synthesis (e.g. actinomycin and cycloheximide), and more recently the use of immunologic and isotope techniques, demonstrated the

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occurrence and importance of this type of adaptation (see Schimke and Doyle 1970 for a review of this subject).

Work performed by Behrisch and his colleagues on numerous animals, including diving mammals (pers. comm.), hibernators (Behrisch 1975, Behrisch and Johnson 1974), and marine ectotherms (Behrisch 1974), has demonstrated that these animals do produce variants of regulatory enzymes of the glycolytic pathway in response to change in environmental conditions (usually temperature). In some cases, rather than producing variants in response (or anticipated response) to changes, the various forms are present simultaneously, but due to their different properties, usually involving enzyme ligand affinities, are maximally active under different specific conditions. The variants are almost never active at their maximal rates (V_{max}) under normal physiological conditions. Rather the dominant isozymes under each condition have evolved Km values that are close to the physiological concentrations of their substrates under these conditions. This allows for tight regulation of enzyme activity in a narrow range of substrate concentrations, because of the hyperbolic, or sigmoidal form of the enzyme substrate saturation curves.

It has been reasonably well documented that intracellular concentrations of various ions and metabolites are altered in response to changes in the environment, and in particular during adaptation to temperature. There is no

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doubt that changes in the concentrations of these substances, which may act as cofactors, positive or negative modulators, or cause changes in intracellular pH, are capable of affecting enzyme activity. Behrisch (1978) demonstrated that the activity and allosteric properties of pyruvate kinase from Arctic ground squirrels *(Spermophilus parryi)* are altered by changes in levels of various cations and metabolites, and that concentrations of cations in the liver of these animals do change throughout the year in a cyclic fashion. It is interesting that with all of the other changes taking place, the blood pH of hibernators does not respond to temperature as it does in most other organisms in which pH increases as temperature decreases, thus preventing the onset of acidosis. In the hibernator's blood, pH either stays constant or changes only slightly (Malan et al. 1973), but in the liver the pH change parallels that seen in other organisms (Malan et al. 1976).

It is evident that molecular changes in the metabolic apparatus do occur in response to changes in environmental conditions. These changes allow for continued efficient functioning of an organism under extreme conditions.

If any kind of regulation of the HMP is intrinsic to the pathway, it probably lies in the two initial dehydrogenase reactions catalyzed by G6PD and 6PGD. Even if there is no intrinsic regulation of the pathway, in terms of the classical allosteric-type regulation, there is a regulatory

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effect on the pathway produced by changing concentrations of metabolites, cofactors, and enzymes, and for the reasons presented earlier it has long been felt that these two enzymes play a key role. As pointed out earlier, G6PD would be expected to catalyze an irreversible reaction because of its high $-\Delta G°$ value, and 6PGD a reversible one. This would seem to implicate G6PD as the committing, if not regulatory, step in the HMP. However $\Delta G°$ values by themselves do not tell us anything about the physiological condition of these enzymes. Greenbaum et al. (1971) has calculated the equilibrium constants (Keq), and the mass action ratios for both of these enzymes from rat livers. These data, in addition to data on metabolite profiles derived from crossover plots, show that indeed the G6PD reaction is poised in the direction of 6PG formation, while the 6PGD reaction is almost at equilibrium. It must be pointed out that these data certainly do not rule out any role for 6PGD as an enzyme active in controlling flux through the pathway, and other evidence, which I will cover later, indicates an important role.

A great deal of work has been done purifying and characterizing these enzymes from a large assortment of organisms ranging from bacteria to fish and mammals. The activities of these enzymes from the liver of rats and brook trout *(Salvelinus fontinalis)*, subjected to a variety of dietary regimes and stresses (including starvation), have

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been studied extensively and appear to fluctuate in response to the changes. In response to a diet containing excess carbohydrate, the activity of the dehydrogenases in rats may increase up to ten-fold (Tepperman and Tepperman 1958a), and in brook trout starvation causes a significant decrease in their activity (Yamauchi et al. 1975). Temperature acclimation of poikilotherms has also been shown to affect the activity of the HMP by modulation of the capacity of these two enzymes. The activity of both enzymes from hepatopancreas of the blue crab *(Callinectes sepidus)* (Robert and Gray 1972a), and brook trout (Yamauchi et al. 1975) increased by 25-50% following cold acclimation from 20 to 10°C, and 15 to 5°C respectively. In contrast, their activities in rats decrease 50-75% on exposure to 5°C (Hannon 1960).

Investigation of modulation of these enzymes has revealed a large number of compounds that act as inhibitors under *in vitro* experimental conditions on purified enzyme. The majority of the work on 6PGD from mammals has been done on the enzyme from sheep liver (Dyson and D'Orazio 1971, 1973, Villet and Dalziel 1972) and rats (Proscal and Holten 1972), with some work done on a variety of other species (Toews et al. 1976, Ollson 1972). The results of this work reveal some interesting interspecific differences in enzyme characteristics, some of which may be related to differences in feeding habits and others not having any

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clear basis. A majority of these modulators probably have no physiological significance because of their extremely high inhibition constant (Ki) values in relation to actual concentrations in the cytosol, but a few of them are probably of physiological importance and are worth considering in terms of possible significance to the "fine" control of HMP activity.

NADPH has been shown to be a potent inhibitor of both 6PGD and G6PD in all organisms. The inhibition of 6PGD has been termed competitive with respect to $NADP^+$ by Proscal and Holten (1972), with a Ki of 20 μM in rats. Villet and Dalziel (1972) found the enzyme from sheep to have a Ki of 2.4 μM with respect to $NADP^+$, but refer to the inhibition as simple end-product inhibition. This would seem to be the more accurate explanation, and would function by stopping the reaction as products build up and equilibrium concentrations are reached. However, Proscal and Holten's (1972, 1976) work on 6PGD and G6PD, and that of Krebs and Eggleston (1974) with G6PD, have both shown that it is the ratio of $[NADPH]/[NADP^+]$ that is important in the inhibition of these enzymes. They have also shown that the concentrations of these metabolites calculated to be present in the cytosol of the rat liver by Veech et al. (1969) are almost completely inhibitory to both enzymes. Sapag-Hagar et al. (1973) reported that the two dehydrogenases are inhibited to different degrees by $[NADPH]/[NADP^+]$ ratios, and that

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6PGD activity would be 1/100 that of G6PD *in vivo*. This would cause a serious imbalance, placing 6PGD in the position of causing a bottleneck in the pathway, and thus possibly controlling the flux through the pathway. The findings of Holten et al. (1976) do not substantiate this, but suggest that the activities of the enzymes are extremely well balanced. Complicating matters further, Whitten and Klain's (1969) data from the 13-lined ground squirrel *(Spermophilus tridecemlineatus)* show that in the hibernating and arousing animals these enzymes are well balanced, while in the nonhibernating animals 6PGD appears to have a lower activity than G6PD. Results from Behrisch's laboratory (pers. comm.) on the Arctic ground squirrel show the same relationship as Whitten and Klain's data.

Proscal and Holten (1972) view this inhibition as a control of HMP flux by NADPH utilizing pathways (i.e. lipogenesis) in a manner similar to the regulation of energy charge and glycolysis (Atkinson 1968). An increase in lipogenesis would rapidly oxidize NADPH, thus decreasing the $[NADPH]/[NADP^+]$ ratio and causing the deinhibition of the dehydrogenases. Thus the $[NADPH]/[NADP^+]$ ratio would again increase to the inhibitory steady state level.

Krebs and Eggleston (1974) take a different approach to the problem. They see the regulation of G6PD activity as a matter of deinhibition, thus allowing the HMP pathway to remain active when lipogenic activity is not oxidizing

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NADPH. Activity at these times may be important in producing precursors for pathways other than lipogenesis. It may also be essential at times for the pathway to remain inactive when substrate is abundant and equilibrium favors the reaction. In view of this possibility they began a search for compounds that counteract the inhibition by NADPH in rat liver, and found that AMP and the oxidized form of glutathione (GSSG) were the only two that had significant effects. AMP was found ineffective at physiological concentrations, while GSSG was highly effective under physiological conditions in all rat tissues except lactating mammary glands. GSSG deinhibition was also found effective in human tissues, but not in bacteria. Recently Rodriguez-Segade et al. (1979) have shown this same effect on 6PGD and G6PD from rat liver and mussel hepatopancreas. The mechanism of this effect is not clear, but it appears to require a cofactor (the identity of which is still a mystery), and does not seem to require the removal of NADPH by the glutathione reductase reaction.

Fructose-1,6-bisphosphate (FBP) has been demonstrated to be a competitive inhibitor of 6PGD from sheep liver (Dyson and D'Orazio 1971, 1933a). These authors suggest that this may be of regulatory significance, under physiological conditions, in controlling the entrance of G6P into the HMP. FBP concentrations would increase with an activation of glycolysis, which would stimulate PFK and PK, in

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addition to its inhibition of 6PGD, thus further driving glucose towards the glycolytic pathway. Proscal and Holten (1972) were unable to find any inhibition by FBP in their work on rat liver 6PGD, but offered no explanation for this difference.

Recently Beitner and Nordenberg (1979) showed that glucose 1,6-bisphosphate (G 1,6-P), an intermediate in glycogen metabolism, is a potent inhibitor of 6PGD from rat liver, muscle, testis and fat pads. This compound is also known to be a strong activator of PFK and PK, and a potent inhibitor of hexokinase, thus acting as a possible regulator of glucose entrance into the HMP. This would be especially true when glucose levels are low and glycogen is being metabolized through glycolysis. This recent finding offers some interesting evidence for a theory of control of HMP activity by 6PGD, but is in need of more attention and work. Whatever the actual role played by these various modulators of HMP dehydrogenases in regulation of flux through the pathway, these, or some other as yet unknown compounds, must necessarily act to give the cell some sort of fine control over the apportioning of glucose between glycolysis and the HMP in response to rapid changes in glucose levels and energy state.

Although these kinds of modulation of enzyme activity are certainly involved in the almost instantaneous adjustments of fine control in response to the rapid changes

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occurring almost constantly in cells, they are probably not the major means of adaptation to long term environmental changes. As mentioned earlier, the levels of HMP dehydrogenase activity from rat liver have been shown to decrease with starvation, and increase over ten-fold after refeeding with a high carbohydrate diet. Similar responses were also discussed in poikilotherms with respect to starvation and acclimation to cold. Further, in studies of both HMP dehydrogenases from hepatic tissue of the 13-lined ground squirrel (Whitten and Klain 1969), and of 6PGD from hepatic tissue of the Arctic ground squirrel (Hannon and Vaughan 1961) it has been shown that their activities are much lower in the hibernator than in the nonhibernating animal. At this point we can essentially think of hibernation as a set of adaptations to cold stress and starvation.

Are these metabolic responses to environmental changes due to quantitative or qualitative adjustments of the enzymes? Behrisch and Percy (1974) demonstrated that two variants of 6PGD exist in adipose tissue of the spotted seal *(Phoca vitulina)*. The two variants have different properties and responses to temperature, and are found to have different activities in different parts of the body. The deep adipose tissue is essentially homeothermic and has a high activity of the variant with a strong dependence on temperature, while the flipper is essentially heterothermic and has a greater activity of the variant that is relatively

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independent of temperature. Thus we see that qualitative adjustments of these enzymes can and do occur. A recent study by Dao et al. (1979) supports the possibility of qualitative changes playing a role in the activity changes of HMP dehydrogenases in rats subject to starvation and refeeding. They purified a variant of G6PD from rat liver that has a specific activity about three times that of the other lower molecular weight forms found previously. They postulate that this form could possibly play a role in the hyperlipogenesis and increased activity of G6PD after carbohydrate refeeding, but further work needs to be done on the activation and inactivation of these forms before we can begin seriously to develop a mechanism.

The studies of Robert and Gray (1972a, b) on HMP dehydrogenases from the blue crab, in which they looked at the increases in activity associated with cold acclimation, do not support a qualitative change in the enzymes. They showed that Km values, and isozyme profiles of the enzymes of cold and warm acclimated animals were not significantly different, and concluded that the mechanism involved in the greater activity of cold acclimated animals was probably an increase in the amount of enzyme present.

The greatest amount of work in this area has been done on rats subjected to starvation followed by refeeding with a high carbohydrate diet, and on rats subjected to insulin treatments. Starvation brings about a decrease in

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lipogenesis accompanied by a similar decrease in NADPH production by the HMP dehydrogenases. Activity is restored to normal levels within 12 hours of refeeding with a high carbohydrate diet. After 48 hours HMP activity is extremely high and still climbing (Tepperman and Tepperman 1958b). Insulin has also been shown to increase the activity of the HMP dehydrogenases over the same time course (Steiner and King 1964), but the effect is caused by the hormone's ability to stimulate the appetite and increase carbohydrate intake (Rudack et al. 1971). Investigators have long felt that these changes in activity were controlled by regulating the rate of enzyme synthesis, which was demonstrated in the case of dietary changes by Johnson and Sassoon (1967). Other investigators (Mack et al. 1975) using drugs that block protein synthesis have demonstrated that the increases in G6PD and 6PGD activities brought about by carbohydrate feeding are mediated by a rapid change in RNA synthesis. The majority of evidence strongly points towards a quantitative change as the major mechanism of adapting to long term environmental changes by these enzymes, and this theory has been widely accepted. The evidence pointing towards qualitative changes in the enzymes should not be viewed as taking anything away from the accepted theory. Rather, I believe that this brings out the point made earlier that regulation, or control, of enzymatic pathways is not a simple "unimechanistic" system, but a

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concerted effort employing a variety of mechanisms acting simultaneously at several levels of cellular functioning.

Ecological and Physiological Considerations of Hibernation

Hibernating animals are able to withstand long periods of low temperature and little (or no) intake of food by entering a state of reduced metabolic activity accompanied by a pronounced bradycardia, a large drop in respiratory rate, and a lowering of body temperature. The Arctic ground squirrel *(Spermophilus parryi)* exhibits this behavior to an extreme degree. It hibernates for up to eight months (Hock 1960) with a concomitant reduction of metabolism well below its normothermic rate, and decrease in body temperature to as low as 0°C.

During extended periods of hibernation the animal arouses every 10 to 14 days, and raises its body temperature to 37°C through increased metabolic activity. Arousal takes about 90 minutes, and the animal stays aroused for approximately 18 hours. Galster and Morrison (1975) showed that these short periods of arousal are accompanied by an increase in glucose reserves, which are depleted during the periods of torpor. Glucose metabolism is not a major source of energy during hibernation, but certain tissues are preferentially dependent on carbohydrate metabolism as an energy source (Sokollof 1960). The primary source of energy for maintenance of physiological processes during hibernation

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is lipid metabolism. This is shown by respiratory quotient values (RQ=the ratio of the volume of CO_2 produced to the O_2 consumed per unit time) and changes in body composition (Lyman and Chatfield 1955). Lipid synthesis is almost entirely shut down during hibernation (Tashima et al. 1970), requiring the animal to use fat that was accumulated before entering the hibernation state. Wilber and Musacchia (1950) looked at the kinetics of lipid turnover in Arctic ground squirrels, and demonstrated that the level of fat metabolism is very high in early summer and falls off dramatically as winter approaches. Thus, it is not surprising to find that most hibernators become extremely fat during the summer when food is readily available, and enter hibernation in a state of extreme obesity.

We know that a large proportion (approximately 50%) of the NADPH required in lipogenesis is produced in the reactions catalyzed by the two HMP dehydrogenases, G6PD and 6PGD, and that changes in their activity probably play a role in control of lipogenesis. In an earlier part of this discussion we saw that the activities of these enzymes decrease in response to cold acclimation (Hannon 1960), and a reduced food intake (Tepperman and Tepperman 1958a) in rats. The activities of these enzymes from hepatic tissue of hibernating ground squirrels, which are subject to extremes of these same conditions, respond in the same manner (Whitten and Klain 1969, Hannon and Vaughan 1961).

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In the summer active squirrels, when temperatures are warmer and there is an abundance of food, the enzymes show much increased activities and the activities under all conditions are much higher than in rats (Hannon and Vaughan 1961). Considering the requirement of lipid synthesis for NADPH, this last point strongly suggests a much greater capacity for lipogenesis in the livers of squirrels than rats. This is substantiated by the prodigious amounts of fat put on by squirrels in the late summer. The lower activity found with the onset of hibernation, a condition of reduced food intake and low body temperatures, correlates well with previous findings in the rat, a nonhibernator. It is of interest to note the work of Ollson (1972) in which G6PD activity and lipogenesis in the liver of the European hedgehog *(Erinaceus europaeus)* are shown to increase with the onset of hibernation. This would not be surprising if the hedgehog were known to hoard food and eat during its cyclic periods of arousal, but as with the ground squirrels these animals do not eat during hibernation. Golden hamsters *(Mesocricetus auratus)* are hibernators that do eat during active periods of the hibernation cycle, and lipogenesis in these animals is almost completely blocked during hibernation (Denyes and Carter 1961). To my knowledge HMP dehydrogenase activities have not been examined in these animals, but Denyes and Carter (1961) proposed that "alterations in the enzyme

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glucose-6-phosphate dehydrogenase" are involved in the lipogenic block. The results from these three different hibernators raise some questions as to the role of increased carbohydrate intake in the induction of HMP dehydrogenase synthesis.

In view of all of these factors, and as part of this laboratory's ongoing research into metabolic control of hibernation at the molecular level, research was done on 6PGD to determine: 1) whether isozymes of the enzyme exist in the liver of the ground squirrel, and if so, whether or not they are produced in response to the stresses of hibernation; and 2) the kinetic characteristics and inhibitors of the forms of the enzyme that are found. This information might reveal the extent of the roles of qualitative and quantitative changes in adapting to environmental stresses, and lead to an understanding of the regulation of flux through the hexose monophosphate pathway.

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METHODS AND MATERIALS

Experimental Animals

Mature Arctic ground squirrels *(Spermophilus parryi)* of both sexes were live-trapped along the Richardson Highway in the Alaska Range of south-central Alaska during the spring and fall. The animals were transported to the University of Alaska, Fairbanks, where they were held in controlled environment chambers of the Combined Animal Facility. Hibernating animals were kept at 5°C with no light during the fall and winter. Summer active animals (this term will be used interchangeably with "nonhibernating animals" throughout this paper) were kept at 15°C with a controlled light cycle (12 hours dark/12 hours light) during the spring and summer. The animals were fed Purina laboratory chow, water and carrots *ad libitum*.

Tissue Preparation

Six nonhibernating, and four hibernating animals were killed by a sharp blow at the rear of the skull. The livers were removed immediately, and either used at once or frozen in liquid nitrogen and stored at -90°C until needed.

The 6-phosphogluconate dehydrogenase (6PGD) used in determining kinetic parameters was prepared by a modification of the procedure described by Dyson et al. (1973). All preparations were done at ice water temperature and reagents

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were kept at approximately 0°C. Minced liver was homogenized at medium speed in four volumes (v/w) of cold 50 mM KCl for 30 seconds, with a Polytron model PCU-2 high speed homogenizer. The homogenate was stirred vigorously for one hour in the cold room. This suspension was centrifuged for 35 minutes at 11,000 g (0-5°C) in a Sorval RC2-B centrifuge (SS-34 rotor). The sediments were discarded.

The supernatant fraction was brought to 45% saturation with solid ammonium sulfate and stirred for two hours in the cold to promote protein precipitation. The turbid suspension was centrifuged for 30 minutes at 11,000 g and the sediments (containing proteins) were discarded. The supernatant fluid was brought to 70% saturation with ammonium sulfate and stirred in the cold until all of the ammonium sulfate was dissolved. This turbid solution was centrifuged for 30 minutes at 11,000 g. The precipitate (containing 6PGD) was dissolved in 1/10 the volume of the original extract of cold 50 mM phosphate buffer (pH 6.3).

This solution was dialyzed overnight in the cold against two changes of 400 ml of 15 mM phosphate buffer (pH 6.3) to remove ammonium sulfate (an inhibitor of the enzyme), and centrifuged for 10 minutes at 11,000 g. The supernatant was frozen in 2-4 ml samples until needed.

Tissue used in electrofocusing experiments was left in a crude state to lessen the chances of denaturation occurring while undergoing electrofocusing. The minced

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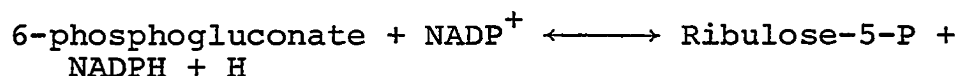
tissue was homogenized with a Polytron homogenizer at medium speed in four volumes of cold 10 mM phosphate buffer (pH 6.4) for 30 seconds, and centrifuged for 10 minutes at 30,000 g in a Sorvall RC2-B centrifuge (SS-34 rotor). The supernatant was stored frozen in the same manner as the other samples until used in determining isoelectric points.

Assay Procedures

Enzyme activity was measured spectrophotometrically, using either an Hitachi Perkin-Elmer model 111 UV-Vis spectrophotometer equipped with a Bausch and Lomb VOM 11 log linear recorder, or a combined Gilford model 2000 multiple sample absorbance recorder-Beckman DU monochromator unit. A continuous recording was made of absorbance at 340 nm as the reaction occurred.

The reduced nicotinamide adenine nucleotides have strong absorbance at 340 nm, while the oxidized forms do not. The activity of the following reaction catalized by 6-phosphogluconate dehydrogenase:



$$\text{6-phosphogluconate} + NADP^{+} \longleftrightarrow \text{Ribulose-5-P} + NADPH + H$$

is directly estimated by the reduction of $NADP^{+}$. Thus the enzyme activity is measured as the change in absorbance with time at 340 nm.

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The assay medium was prepared as in Bergmeyer (1974), and contained in a total volume of 2.92 ml,

Assay medium	Concentration in assay
2.50 ml Triethanolamine (0.1 M, pH7.6 at 25°C)	85 mM
0.20 ml 6-phosphogluconate,Na salt(15 mg/ml)	2.7 mM
0.10 ml $NADP^+$,Na salt(10 mg/ml)	0.4 mM
0.10 ml $MgCl_2$(0.1 M)	3.4 mM
0.02 ml Enzyme solution	

All of the reagents were obtained from Sigma Chemical Co., St. Louis, Missouri. This was the basic assay medium, but the concentrations of substrates were varied depending on the experiments being performed. Putative modulators were added as indicated. The reactions were initiated by the addition of $NADP^+$. Assay temperatures were regulated with a constant temperature bath and circulator, and the Triethanolamine (TEA) buffer was kept at the assay temperature at all times. The assay temperatures used were 5, 15, 26 and 37°C, covering the physiological temperature range experienced by ground squirrels during a yearly cycle of activity and hibernation.

The pH of the liver of the hibernator has been shown (Malan et al. 1976) through intracellular recording to be unlike that of other tissues from hibernating animals, in

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that its pH varies with temperature in the same manner as is found in blood (Reeves et al. 1977). In order to keep experimental parameters as close to physiological conditions as possible, all assays were performed at the physiological pH associated with the assay temperature. To facilitate this procedure TEA was chosen as the buffering medium and all reagents were made in this solution. In this context, I showed that the pH of TEA is temperature dependent and this variation with temperature is the same as that found in the liver of the hibernator (Fig. 4). The pH was adjusted at 25°C with NaOH or HCl, as necessary, and a Corning Model 12 Research pH meter.

HEPES (N-2-hydroxyethylpuperazine-N-2-ethanesulfonic acid), a temperature insensitive buffer, was used in place of TEA for determination of the pH profiles of the enzyme. pH was adjusted with NaOH or HCl to range from pH 6.5-8.5. All assays for enzyme activity were measured in triplicate for at least two minutes, and each experiment was performed at least two times.

Electrofocusing

Electrofocusing is a means of separating proteins and discerning the presence of enzyme variants based on the isoelectric point (pI) of the proteins. The solution containing the protein(s) in question is loaded onto a sucrose density gradient with a solution of ampholytes consisting

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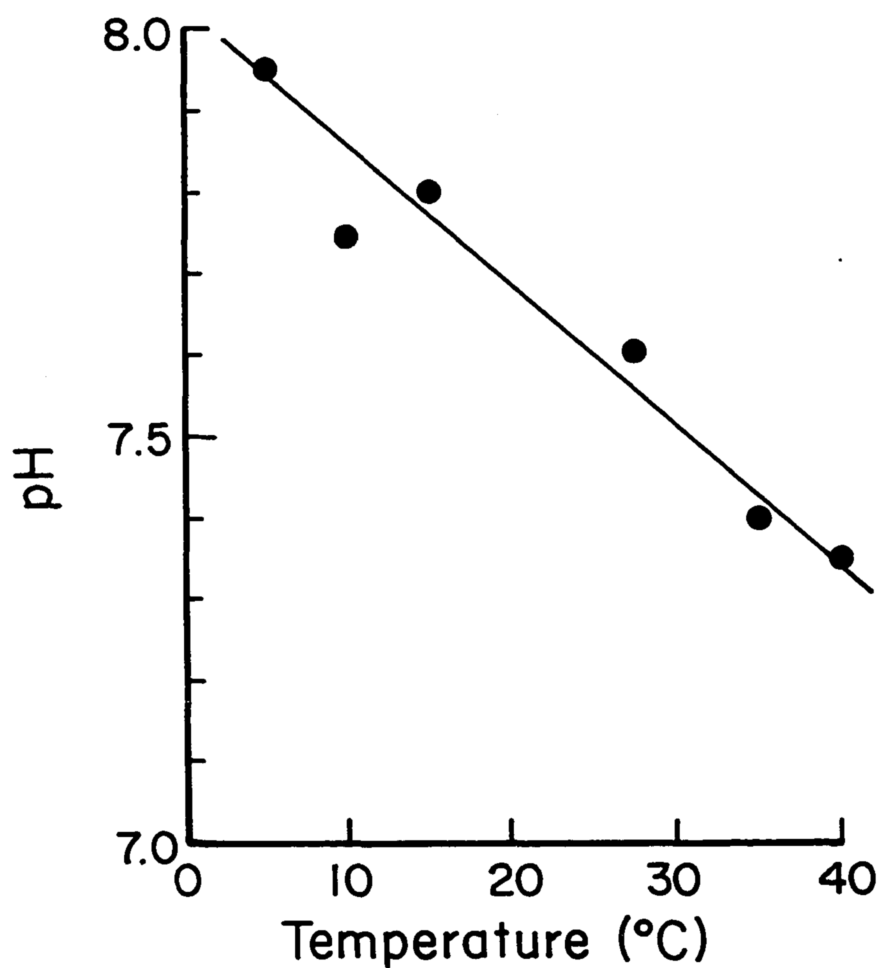

Fig. 4. Effect of temperature on the pH of 0.1 M Triethanolamine buffer.

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of many aliphatic polyamino-polycarboxylic acids. These ampholytes have a high buffering capacity in comparison to the proteins. The sucrose density gradient is a non-ionic solution serving to stabilize the column against thermal convection during the electrofocusing. When a voltage is applied across the column the ampholytes migrate to the points where they are electrically neutral, thus forming a pH gradient. The protein samples eventually migrate to the pH in the gradient at which they are electrically neutral, thus becoming separated by their isoelectric points. Photometric assays are used to determine the fraction containing the active enzyme. The pI is determined by measuring the pH of this solution.

Electrofocusing was performed as in the LKB 8100 Ampholine instruction manual using an LKB 8100 Electrofocusing unit and constant temperature circulating water bath. The 110 ml column and corresponding gradient mixer were used in all experiments. The column was set up with the anode at the bottom and cathode at the top, and run at 400 V for 24-56 hours, or until the current had stabilized at less than 0.5 mA. The column was drained at approximately 1 ml/min, and 2 ml samples were collected using an LKB 7000 UltroRac fraction collector.

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The pH was measured with a Beckman Expandomatic SS2 pH meter. The samples were kept on ice and assayed for 6PGD activity at room temperature (25°C) and pH 7.6, as described in the preceding section.

Electrofocusing runs were conducted on tissue from one hibernating and one nonhibernating animal over pH ranges of 3.5-10 and 4-6.

Presentation of Experimental Results

Enzyme activities from the electrofocusing and pH optima experiments are presented either as the change in optical density per minute (ΔOD/min), or as percent maximum activity (%V_{max}).

Enzyme activity from the kinetic studies is expressed as International Units per gram frozen tissue (U/g) and Units per milligram protein (U/mg). One International Unit (U) of enzyme activity is that amount of enzyme catalyzing the formation of 1 μmole of product per minute. Protein concentrations of the tissue preparations were determined by the micro-biuret technique, a modification of the biuret (Layne 1957). Units of enzyme activity were calculated from the ΔOD/min using the molar extinction coefficient for NADPH (E_{340}=6.22cm^2/μmole).

The kinetic parameters, Km (the substrate concentration required for an initial velocity of 1/2V_{max} and V_{max}

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(maximal velocity when all substrates are present at saturating concentrations) were derived by fitting experimental data to the hyperbolic form of the Henri-Michaelis-Menten equation:

$$v=(V_{max}*[S]/(Km+[S]) \qquad (1)$$

([S]=substrate concentration, v=velocity), using the least-squares method of Cleland (1967) on the University of Alaska Honeywell computer system. Statistical data are presented as standard deviations, and were also calculated using Cleland's method (1967). This method was used in place of the usual linear transformations (Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee), because of deficiencies in these other methods. The Eadie-Hofstee form has been shown to be the most accurate of these methods, and to call more attention to points deviating from the theoretical linear relationship than the other forms (Segel 1976). A point that is often overlooked in using this formula is that the Eadie-Hofstee plot (v vs. v/[S]) has no independent variable, and thus should not be used in a linear regression. The Cleland method appears to be as powerful while still being mathematically valid.

Types of inhibition were determined from Lineweaver-Burk (1/v vs. 1/[S]) linear transformation plots at varying concentrations of the putative modulators. The competitive inhibition constants, Ki (the concentration of inhibitor,

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[I], that doubles the slope of the 1/v vs. 1/[S] plot), were derived from a replot of the slopes of the (1/v vs. 1/[S]) plot vs. [I]. This replot produced a straight line with equation:

$$\text{Slope } (1/v \text{ vs. } 1/[S]) = ((Km/V_{max}*Ki))*[I]) + Km/V_{max} \quad (2),$$

from which Ki is calculated.

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RESULTS

Electrofocusing

Electrofocusing experiments were performed on liver extracts from both hibernating and nonhibernating Arctic ground squirrels. The results of these experiments are presented in Figure 5, and show that 6-phosphogluconate dehydrogenase (6PGD) from the livers of these animals is present in a single form. The single form of the enzyme is the same in the hibernating and nonhibernating animal, and has an isoelectric point of pH 5.15.

Kinetics

Ground squirrel liver 6PGD has a relatively high affinity (as indicated by a low Km), in comparison to many other enzymes (see Lehninger 1975), for its substrates, 6-phosphogluconate (6PG) and $NADP^+$ (Table I and Table II), and substrate saturation data produce hyperbolic curves fitting the Henri-Michaelis-Menten equation (Fig. 6 and Fig. 7).

Comparison of the Km values of the enzyme from hibernating and nonhibernating animals show no significant differences in affinity for $NADP^+$ at 5, 15, 26 and 37°C (Table I). The Km values for 6PG at 5°C show a statistically significant difference (P=0.05) between the hibernator and nonhibernator, but at 15, 26 and 37°C there are no

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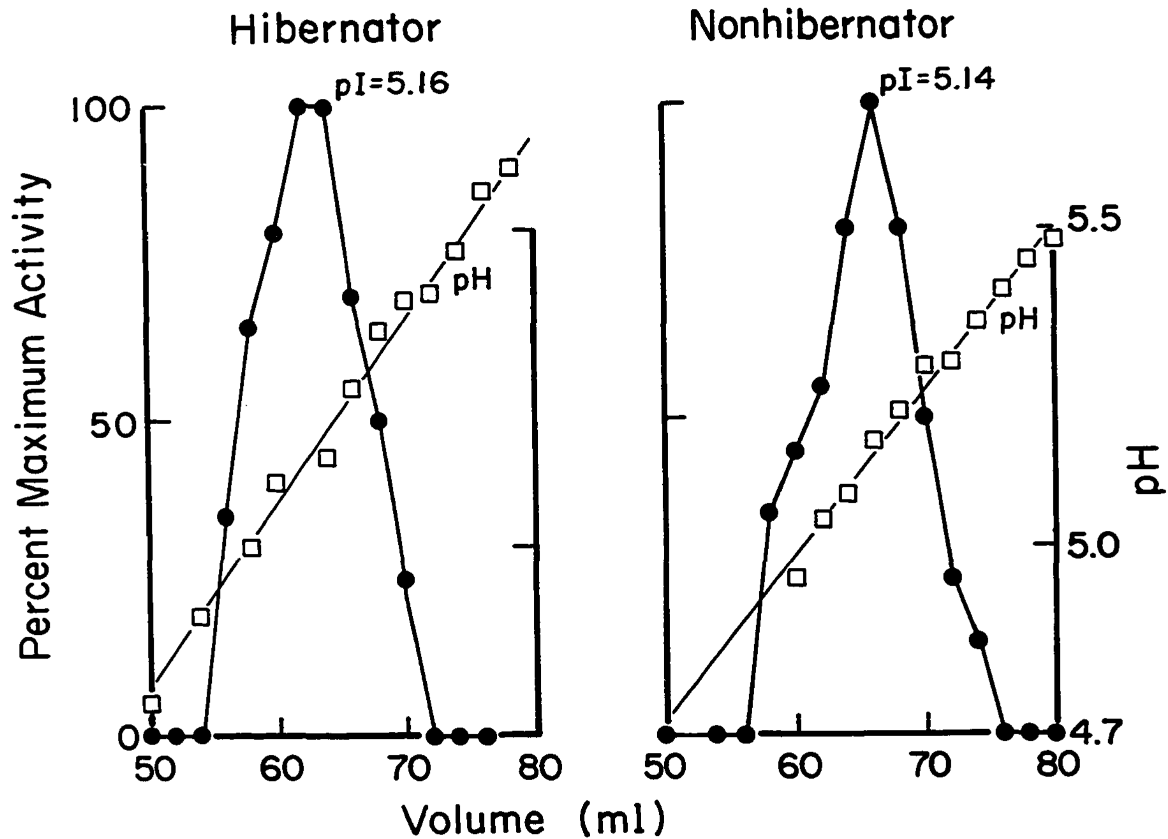

Fig. 5. Electrofocusing of liver 6-phosphogluconate dehydrogenase from hibernating and nonhibernating Arctic ground squirrel. The experiments were run over a gradient of pH 4 to pH 6.

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Table I. The effect of varying temperature on Km for $NADP^+$ and V_{max} of 6PGD from Arctic ground squirrel liver. Km and V_{max} were calculated as described in the text, and statistics are presented as ± standard deviations.

	Temp. (°C)	Km (μM)	V_{max} (U/g)*	V_{max} (U/mg)**
Hibernator: (n=3)***	37	15.40±2.99	1.460±0.080	9.672=0.037
	26	11.36±2.93	0.645±0.048	0.297±0.022
	15	10.91±2.29	0.280±0.016	0.129±0.007
	5	9.12±2.20	0.104±0.007	0.048±0.003
Nonhibernator (n=6)***	37	15.12±1.44	1.760±0.052	0.816±0.024
	26	12.13±1.37	1.023±0.028	0.474±0.013
	15	9.44±0.83	0.356±0.008	0.165±0.004
	5	7.81±1.44	0.128±0.006	0.059±0.003

*Units per gram frozen tissue.

**Units per milligram protein.

***n refers to the number of animals used, whereas the sample sizes used in statistical analyses are the number of complete experiments performed on the extracts from these animals.

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Table II. The effect of varying temperature on Km for 6-phosphogluconate and V_{max} of 6PGD from Arctic ground squirrel liver. Km and V_{max} were calculated as described in the text, and statistics are presented as ± standard deviations.

	Temp. (°C)	Km (μM)	V_{max} (U/g)*	V_{max} (U/mg)**
Hibernator: (n=1)***	37	10.75±1.09	1.040±0.020	0.489±0.009
	26	9.48±0.72	0.556±0.008	0.261±0.004
	15	11.85±1.59	0.272±0.008	0.128±0.004
	5	18.40±1.45	0.120±0.004	0.056±0.002
Nonhibernator: (n=4)***	37	11.91±0.85	1.44±0.024	0.578±0.010
	26	9.22±0.81	0.720±0.016	0.289±0.006
	15	8.16±1.04	0.344±0.008	0.138±0.003
	5	7.19±0.66	0.108±0.002	0.043±0.001

*Units per gram frozen tissue.

**Units per milligram protein.

***n refers to the number of animals used, whereas sample sizes used in statistical analyses are the number of complete experiments performed on the extracts from these animals.

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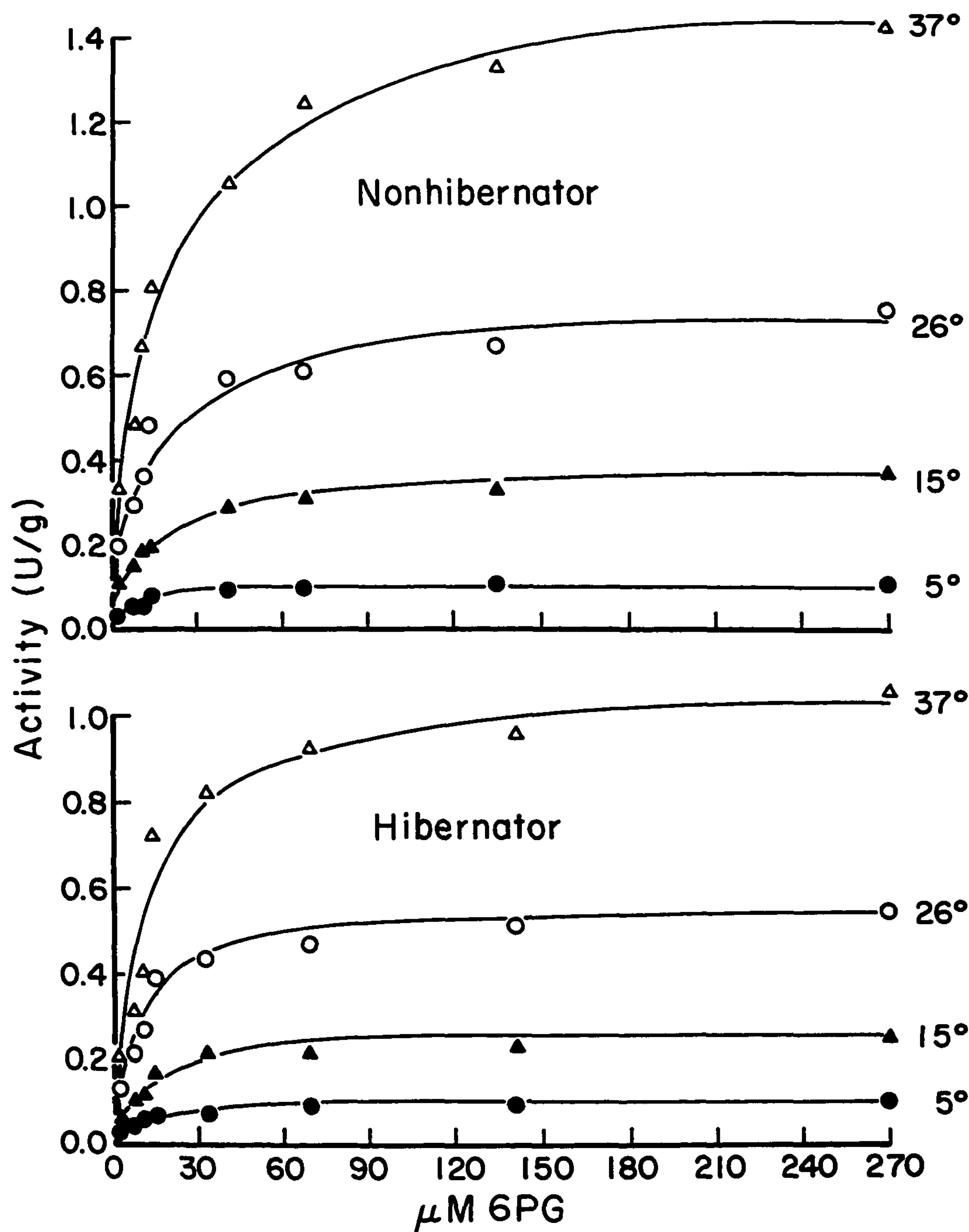

Fig. 6. Substrate saturation curves of hibernating and nonhibernating ground squirrel liver 6PGD with respect to 6PG at 5, 15, 26 and 37°C. Activity is expressed as International Units per gram of frozen tissue (U/g).

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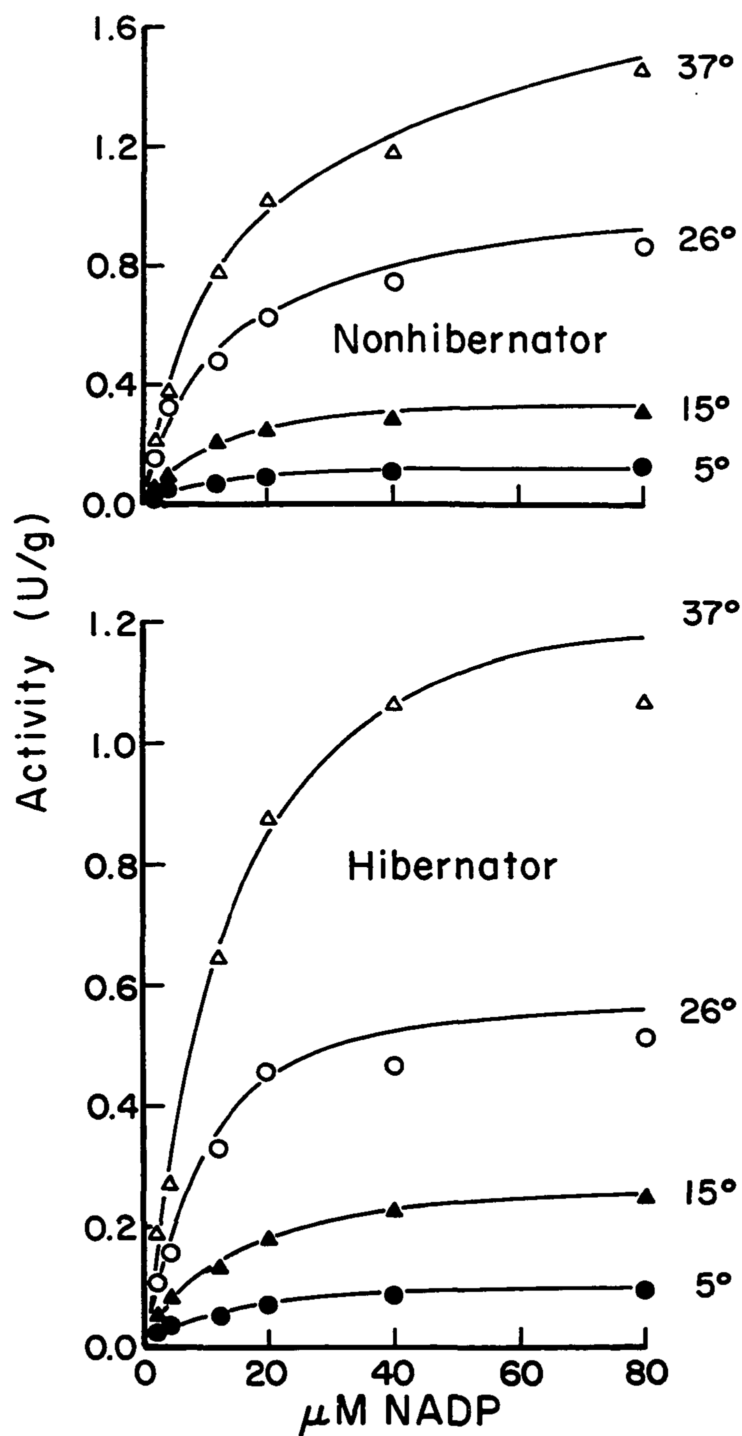

Fig. 7. Substrate saturation curves of hibernating and nonhibernating ground squirrel liver 6PGD with respect to $NADP^+$ at 5, 15, 26 and 37°C. Activity is expressed as International Units per gram of frozen tissue (U/g).

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significant differences (Table II). The difference at 5°C may not be biologically significant since the Km values presented in Tables I and II were calculated from the data obtained in at least two separate experiments, and if the data from the hibernator 6PG saturation curves at 5°C are calculated separately for each experiment the values for Km are 6.98 ± 1.09 μM and 18.23 ± 2.23 μM. The lower value corresponds nicely with the value of 7.19 ± 0.66 μM for the nonhibernator at 5°C. The problem may lie in the limitations of the experimental design. Due to the extremely low levels of activity at 5°C, the majority of points used in determining the kinetic parameters of the enzyme fall near V_{max} (Fig. 6), thus making determination of Km less dependable than those done at higher temperatures. One other source of error that may produce difference in Km values are slight changes in substrate concentrations. Substrates were weighed carefully to 10^{-4} g, but at low concentrations this amount of error may be significant. In the future I would determine the concentrations of all substrates enzymatically in order to avoid this potential problem.

There are no statistically significant differences at P=0.05 between Km values over the temperature range from 5 to 37°C in either the hibernator or nonhibernator, and for either substrate.

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Comparisons of hibernating and nonhibernating values for V_{max} of 6PGD, which are plotted in Figure 6 and Figure 7 and listed in Tables I and II, show approximately a 25% increased specific activity in the nonhibernator at 15, 26 and 37°C, in agreement with earlier work by Behrisch (pers. comm.), and Whitten and Klain (1969). The results of the V_{max} determinations at 5°C, with respect to $NADP^+$ concentration, also show a 25% increase in activity in the nonhibernator, but the results for V_{max} at 5°C, with respect to 6PG concentration, show a decrease of activity in the nonhibernator. I do not believe that this is an accurate result for the same reasons discussed previously in regards to the Km for 6PG of the hibernator enzyme at 5°C.

An Arrhenius plot of log V_{max} as a function of the reciprocal of the absolute temperature of the reaction (1/T) is shown in Figure 8 for the hibernator. Measurements of V_{max} were made between 5 and 37°C, and within this range the energy of activation (Ea) is constant, as can be seen from the constant slope drawn to the points in Figure 8. Ea was calculated to be 52.95 KJ/mole (Table III) by the equation:

$$Ea=2.3*R*(Slope[\log_{10}V_{max} \text{ vs } 1/T]) \qquad (3)$$

(Ea=energy of activation, R=8.31 J mole-1 K-1, T=absolute temperature[K]). The slope and activation energies for the nonhibernator (not shown) were the same as for the hibernator.

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Table III. Activation energies (Ea) and Q_{10} values for hibernating and nonhibernating ground squirrel liver 6PGD. Ea values were calculated from V_{max}.

Temperature range (°C)	Ea (KJ/mole)	Q_{10}*
5-15	52.95	2.22
15-25	52.95	2.11
25-35	52.95	2.01

*Q_{10} values were calculated from Ea ($Ea=2.3RT_2T_1\log_{10}Q_{10}/10$)

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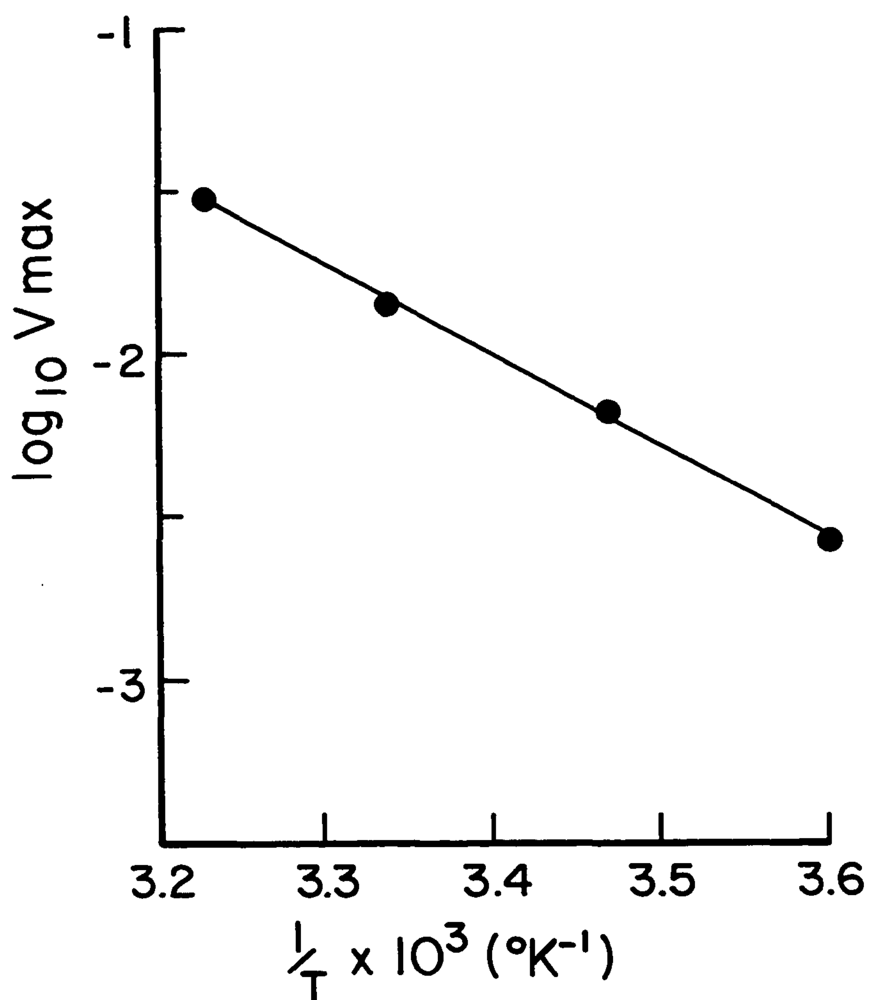

Fig. 8. Arrhenius plot of 6PGD from hibernating Arctic ground squirrel liver. Values are calculated from reaction velocities at saturating concentrations of substrates.

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Based on the electrofocusing and kinetic experiments that have been presented to this point the enzyme from liver of the hibernating and nonhibernating animals appear similar, and thus only the enzyme from either the hibernator or nonhibernator was used in arriving at the following results.

Q_{10} values are listed in Table III and were calculated from Ea. The Q_{10} increases from 2.01 to 2.22 as the temperature decreases from 37 to 5°C, but this small change is probably not biologically significant.

The effects of a number of metabolites, which have been shown to be inhibitors of 6PGD from various other organisms, were tested on 6PGD from nonhibernating ground squirrel liver, and the results are presented in Table IV. Of the six putative modulators tested only NADPH and AMP were found to be inhibitory. Mg^{++} was found to have no effect at all, while ADP, ATP, and FBP showed almost imperceptible amounts of inhibition at very high concentrations. The inhibition by NADPH is competitive for $NADP^+$, as shown in Figure 9, with a Ki of 22 μM at 5 and 37°C (Table IV). AMP is competitive for 6PG (Fig. 10) with a Ki of 2.18 mM at 5°C, and 2.28 mM at 37°C (Table IV).

Activity measurements at 5, 15, 26 and 37°C, as a function of pH, were performed with substrate concentrations approximating those thought to be found in ground squirrel livers. The concentration of 6PG was 30 μM and $NADP^+$ was

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Table IV. Inhibition constants (Ki) for 6PGD from a non-hibernating ground squirrel liver at 5°C (pH 7.9) and 37°C (pH 7.4).

Inhibitor	Ki (mM) 5°C	Ki (mM) 37°C
NADPH	0.02*	0.02*
AMP	2.18**	2.28**
ADP	No inhibition	
ATP	No inhibition	
FBP	No inhibition	
Mg^{++}	No inhibition	

*Determined as competitive with respect to $NADP^+$, and calculated as described in the text.

**Determined as competitive with respect to 6PG, and calculated as described in the text.

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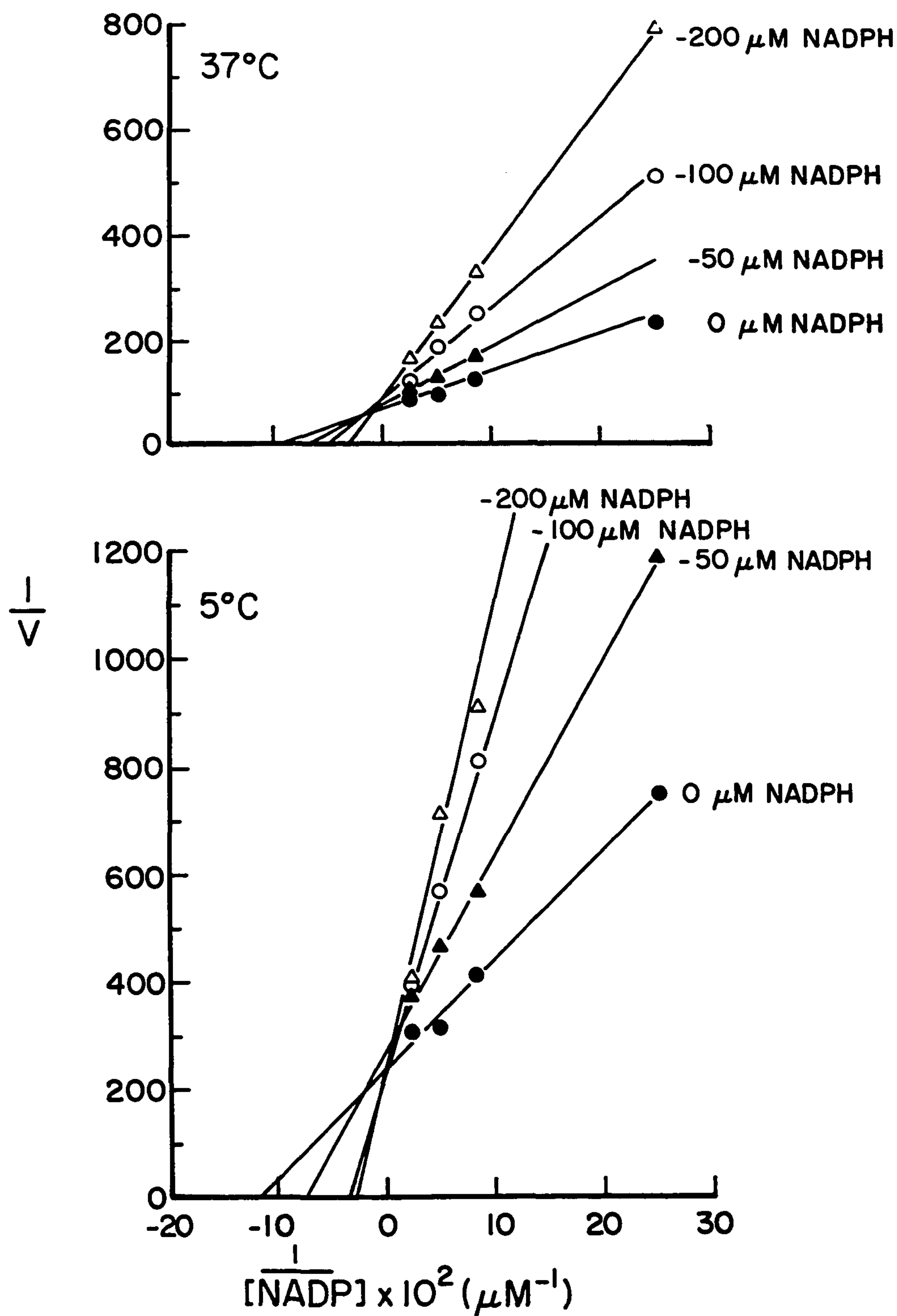

Fig. 9. Effect of NADPH on the activity of nonhibernating ground squirrel liver 6PGD as a function of $NADP^+$ concentration at 5 and 37°C. The concentrations of NADPH, in the assays, were as shown in the figure. All other assay conditions were as described in Methods and Materials.

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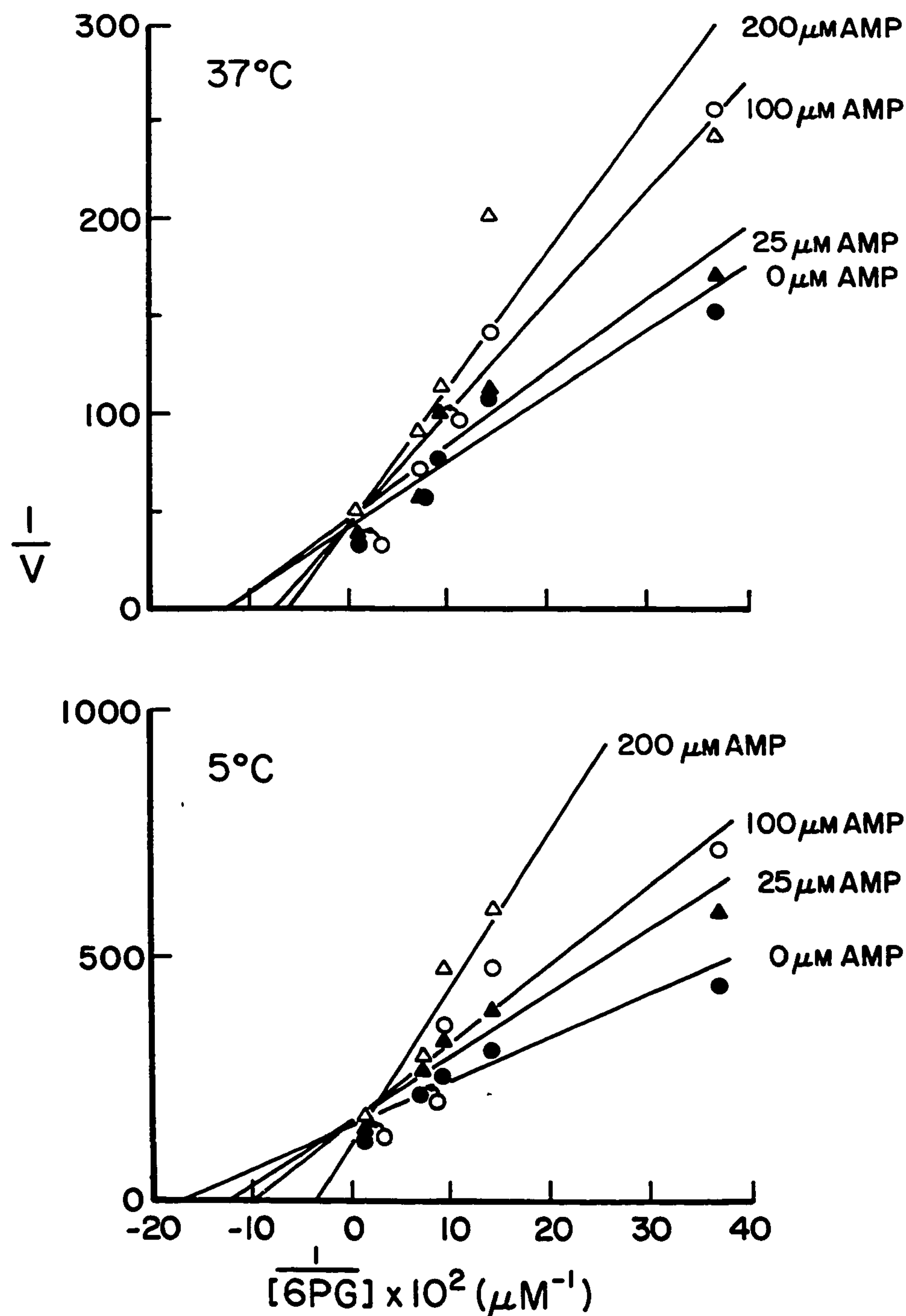

Fig. 10. Effect of AMP on the activity of nonhibernating ground squirrel liver 6PGD as a function of 6PG concentration at 5 and 37°C. The concentrations of AMP, in the assays, were as shown in the figure. All other assay conditions were as described in Methods and Materials.

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8 μM, which are the concentrations found in rat liver (Bergmeyer 1974), and are close to the Km values found for ground squirrels in this study. These measurements are plotted in Figure 11A. There is very little effect of temperature on pH profiles, except possibly at alkaline pH where the lower temperature curves (5 and 15°C) appear to level off while the activities at 26 and 37°C drop off sharply. The pH optimum for the enzyme under physiological conditions is between pH 7.6 and 7.8 at all four temperatures.

Figure 11B shows the effect of pH on activity when all substrates are at saturating concentrations (6PG=2.7 mM, $NADP^+$=0.4 mM), and the enzyme is functioning at maximal velocity. The general shape of the pH profiles at 5, 15, 26 and 37°C show little change from those done at physiological concentrations, except that the pH optimum appears to be shifted towards a more alkaline pH of 8.0. At 5 and 15°C the activities again level off at pH 8.0, but under these saturating conditions the plateau is at what appears to be the range of optimum pH, while at 26 and 37°C the activities drop off at pH greater than pH 8.0.

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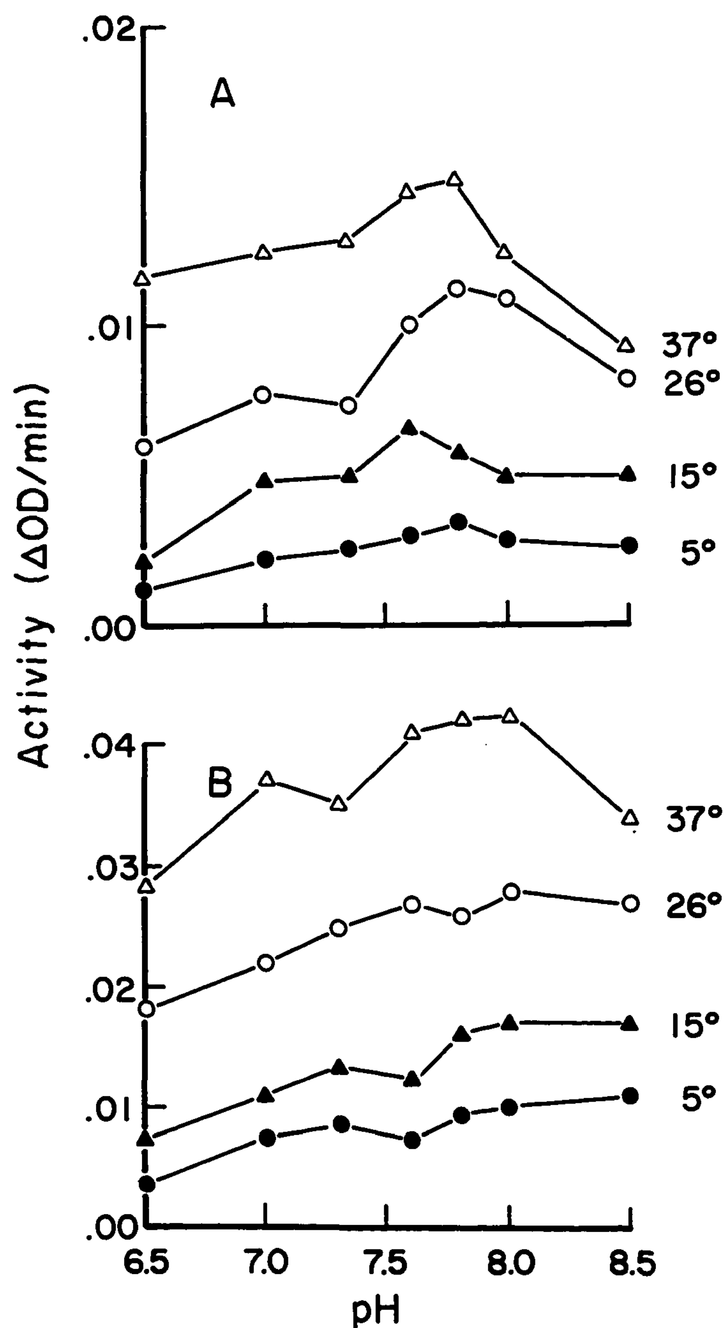

Fig. 11. Effect of pH on activity of hibernating ground squirrel liver 6PGD at 5, 15, 26 and 37°C. A)All substrates present at physiological concentrations: 6PG=30 μM, $NADP^+$=8 μM (as given for the rat in Bergmeyer 1974); B)all substrates present at saturating concentrations. Activity is expressed as the change in optical density per minute (ΔOD/min).

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DISCUSSION

Initial electrofocusing results indicate that there is a single form of 6-phosphogluconate dehydrogenase in liver of the Arctic ground squirrel, and that this form does not differ when the animal shifts from the summer active nonhibernating state to the winter condition of regular cycles of hibernation and arousal. The determinations of the Michaelis constants (Km) support these findings with no significant differences with respect to enzyme-substrate affinity between the hibernator and nonhibernator at temperatures over the physiological temperature range.

It is difficult to say anything definite about the physiological significance of the apparent Km values reported here. Apparent Km describes an important relationship between the concentration of a substrate and the activity of the enzyme. The problem in this case is that we have no data for concentrations of the substrates of 6PGD in the ground squirrel liver. It is probably reasonable to assume that the concentrations in the nonhibernating animal are similar to those found in rats, but in the case of the hibernator we can only speculate. The concentrations of 6PG in the hibernator's liver may be similar to those of the starving rat, but this is unlikely, since although the hibernating ground squirrel is subject to the same environmental stress as the starving rat, it has successfully

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adapted to them so that physiological stress is minimized. Thus, before we can discuss the significance of the Km values for the ground squirrel enzyme and its ability to regulate activity at varying temperatures we need to determine concentrations of the substrates (and other metabolites) in hibernating and nonhibernating animals.

The finding that the hibernating and nonhibernating forms of 6PGD are the same, coupled with the 25% higher specific activity of the enzyme from the nonhibernator, is in general agreement with the trends found by Whitten and Klain (1969) and Behrisch (pers. comm.), with the exception that they found greater differences in activity between the two conditions. The data strongly suggest quantitative changes in enzyme concentration, rather than a qualitative change in the enzyme molecule.

In this connection, quantitative changes in 6PGD concentrations have been implicated in the adaptations of several different organisms to various environmental stresses. Rats adjusting to altered diets and starvation alter the rate of 6PGD synthesis (Johnson and Sassoon 1967), while crabs acclimating to temperature change alter the activity of 6PGD (Robert and Gray 1972a, b). The change in activity of the crab enzyme is clearly not caused by qualitative changes, as demonstrated by comparing isozyme patterns and Km values in a manner similar to that of this

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study. It was concluded that the changes were caused by alterations in enzyme concentration.

It must be concluded from the results of these experiments on ground squirrels that the changes in specific activity between the enzyme from the hibernator and non-hibernator, under equivalent assay conditions, are caused by changes in 6PGD concentrations in the liver.

Not only are there no seasonal modifications in enzyme structure, but there are apparently no compensatory adaptations involved in the response of the enzyme to temperature changes, as demonstrated by the fact that there were no significant differences found between Km values or pH profiles at 5, 15, 26 and 37°C. These results agree with the findings of Dyson, D'Orazio and Hanson (1973) in sheep liver 6PGD. All major changes in the activity of this enzyme that accompany the onset of hibernation appear to be brought about by a combination of seasonal changes in enzyme concentration, and by the effects of reduction of thermal energy accompanying the hypothermic lowering of body temperature. In line with the lack of any special thermal adaptations, the Q_{10} is essentially constant throughout the entire range of temperatures investigated, and allows for a ten-fold decrease in activity when body temperature drops from 37 to 5°C.

It was mentioned earlier that lipid synthesis is entirely shut down during the hibernation season, and thus

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the demand for the NADPH produced by the 6PGD reaction is greatly reduced. Clearly it would be functionally disadvantageous for the animal to maintain a series of reactions, like the hexose monophosphate pathway, which draws glucose away from the energy yielding glycolytic pathway at a time when glucose is scarce. In the hypothermic hibernator the combination of low thermal energy and low 6PGD concentration would effectively shut the pathway down, thus stopping the diversion of G6P through the HMP and needless production of NADPH. During the hibernation period the animal is not always hypothermic, and in fact regularly arouses and becomes normothermic every 10 to 14 days for about 18 hours. If we only consider the effects of thermal energy on enzyme activity, the activity of 6PGD will be increased ten-fold, to within 75% of the activity found in the nonhibernating animal, during these short arousal periods. It is thought that hibernating ground squirrels carry on gluconeogenesis during these periods of arousal (Galster and Morrison 1975) to replace the glycogen that is required by the nervous system. While gluconeogenesis is active other biosynthetic processes, specifically lipogenesis, remain turned off. Since the products of the 6PGD reaction, and the HMP in general, are not needed during these periods, and since catabolic metabolism of glucose lies at the heart of the pathway, it would be extremely

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advantageous if 6PGD remained inactive during these brief, but necessary, periods of arousal.

In devising models of enzyme control, other than those based solely on thermal energy and enzyme concentration, that might allow this enzyme to remain inactive under normothermic conditions, I investigated the effects of various metabolites that may be involved in the fine control of enzyme activity. All of the metabolites investigated had been shown to be modulators of the enzyme in other animals.

Our findings of no effect by either FBP or Mg^{++} on the enzyme are in agreement with those of Proscal and Holten (1972) in rats. Dyson and D'Orazio (1971) report FBP as a potent inhibitor of sheep 6PGD, and cite McLean and Gumaa (1969) as reporting inhibition in rats. Villet and Dalziel (1972), Dyson and D'Orazio (1973) and Dyson, D'Orazio and Hanson (1973) all report that Mg^{++} and other cations activate sheep liver 6PGD. Dyson and D'Orazio also report that ATP inhibits the enzyme, and that the complexing of Mg^{++} and ATP may reduce this inhibition. This might explain the difference in results with Mg^{++}, as we found no inhibition by either ATP or ADP in ground squirrels. In a detailed study of the effects of ionic strength and Mg^{++} on the enzyme from sheep liver Dyson, D'Orazio and Hanson (1973) demonstrated activation by both the cation and increased ionic strength, and state that the contribution of Mg^{++} to the ionic strength was insufficient to

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produce maximal effects on enzyme activity, but that optimum ionic strength of 0.08-0.1 would activate the enzyme to its maximum velocity without Mg^{++}. Since the effects of ionic strength were not considered in this study, or by Proscal and Holten (1972), and the effect of ionic strength on the ground squirrel enzyme is unknown, the possibility that ionic strength was optimum, thus masking the effect of Mg^{++} on activity, can not be ruled out.

The Lineweaver-Burk plots for inhibition by AMP and NADPH (Fig. 10 and Fig. 11) show clearly that AMP and NADPH are competitive with 6PG and $NADP^+$ respectively. Inhibition by AMP may be significant in regulation of glucose entrance into the HMP at certain times, depending on the extent of its concentration in the ground squirrel liver. However, it is unlikely to have much effect during hibernation, and even less likely during periods when gluconeogenesis is occurring, because the AMP concentrations should not be high at these times.

Extreme competitive inhibition by NADPH, which may be regarded as simple end-product inhibition or as a function of the [NADPH]/[$NADP^+$] ratio, has been shown in 6PGD from all sources investigated, and is generally thought to be the major regulator of both 6PGD and G6PD. In all of these other organisms the inhibition by this molecule has been so great under physiological conditions that without other mitigating circumstances the enzyme is shut off completely

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in vivo. The Ki for 6PGD from ground squirrels of 20 μM is the same as in rats (Proscal and Holten 1972) where an [NADPH]/[$NADP^+$] ratio of eight is almost completely inhibitory (Krebs and Eggleston 1974). The *in vivo* ratio in rats is of the order of 100 (Veech et al. 1969).

In the ground squirrel a ratio of five causes over 50% inhibition, and although any estimates of metabolite concentrations are speculative I would again suspect that in the nonhibernator concentrations are similar to those in the rat. In the hibernating animal, concentration ratios would likely be of the same order, or possibly slightly higher than in the nonhibernator. The possibility of a higher ratio during hibernation is dependent on two unsubstantiated, but not unlikely, points. The first of these is that the HMP dehydrogenases are not shut off completely *in vivo* and continue producing NADPH, albeit at a greatly reduced rate. Second, the block of lipogenesis, the major NADPH utilizing pathway, is not directly controlled by NADPH levels and remains turned off in the presence of this cofactor, thus causing a slow buildup of NADPH. If the block in lipogenesis is not directly controlled by the availability of NADPH, and is capable of inducing its production as suggested by Tepperman and Tepperman (1961), this could be a mechanism explaining the high 6PGD in the nonhibernator and the low activity in both the hypothermic

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and normothermic hibernator. If we accept this last point we are faced with the problem of explaining the regulation of lipogenesis that is not limited by NADPH, which should be a topic of further study.

The role of oxidized glutathione (GSSG), and its unidentified cofactor, as deinhibitors of the HMP dehydrogenases in rats, humans and mussels was discussed in the introduction. The effect of this compound on the ground squirrel enzyme was not checked in this study, but should be in future work. However, one finds the same deinhibition in such different organisms as rats and mussels, and I should not be surprised to find the same thing in the ground squirrel. If in the future this is shown to be so, and that at physiological concentrations the potent inhibition by NADPH does keep the enzyme shut off, I would propose that GSSG may indeed be acting as an important regulator in the fine control of HMP dehydrogenase activity. Fluctuations in GSSG or its polypeptide cofactor could serve to keep the enzyme shut off during the period of hibernation, and stimulate it during the period of summer fattening. Unfortunately we do not have any values for concentrations of GSSG in ground squirrels. Although Eggleston and Krebs (1974) have found that physiological concentrations would be able to counteract the NADPH

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inhibition in rat liver, they cite the discussion by Veech (1968) on the problems of obtaining reliable tissue concentrations of this compound. The unreliability of their methods made it impossible for them to determine whether the changes in concentration required to produce fine control of the enzyme occur. They did, however, demonstrate seasonal changes, possibly triggered by photoperiod, in the ability of GSSG to deinhibit the enzyme. This would probably be important in an animal like the hibernator that is so dependent on seasonal cycling, and would increase the likelihood of GSSG being one of the primary regulators of the ground squirrel enzyme.

Glucose 1,6-bisphosphate (G 1,6-P) has recently been implicated in the regulation of the HMP pathway. Beitner and Nordenberg (1979) found that this compound, which is produced in the breakdown of glycogen to G6P, is inhibitory to 6PGD from rats, as discussed briefly in the introduction. I did not examine the effects of G 1,6-P on the ground squirrel enzyme, but feel certain that future work will show the same inhibitory effects on it as in the rat. If indeed the compound does have the same inhibitory effects on 6PGD and HK, and activating effects on PFK and PK, as it does in rats, it would be an important regulator of the HMP pathway during hibernation when glycogen is the major source of glucose for the nervous system.

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In considering models for regulation of 6-phosphogluconate dehydrogenase from Arctic ground squirrel liver one must first differentiate between fine control in response to rapid changes in the physiological environment, and the long term coarse control involved in seasonal adjustments to hibernation (Fig. 12). As already discussed, the ground squirrel's major seasonal adaptation of 6PGD is quantitative. The concentration of this enzyme is increased during the active summer period, and activity is decreased during hibernation by a reduction in concentration coupled with a low thermal energy. In addition to these physiological adjustments we know that NADPH is a major inhibitor of 6PGD in these animals, and probably would be able to completely inhibit the enzyme at physiological concentrations. The effect of GSSG on 6PGD from ground squirrels is unknown, but as it is known to counteract the inhibition by NADPH in a wide variety of other organisms, I predict that we will find the same effect in the ground squirrels. I would make a similar prediction in the case of G 1,6-P inhibition, as the accumulation of this molecule would likely occur at the same time as its effect would be most useful.

There are obviously a number of experiments that remain to be done on ground squirrel liver 6PGD before a clear picture of its regulation can be presented. Notable among these are studies of the effects of GSSG and G 1,6-P

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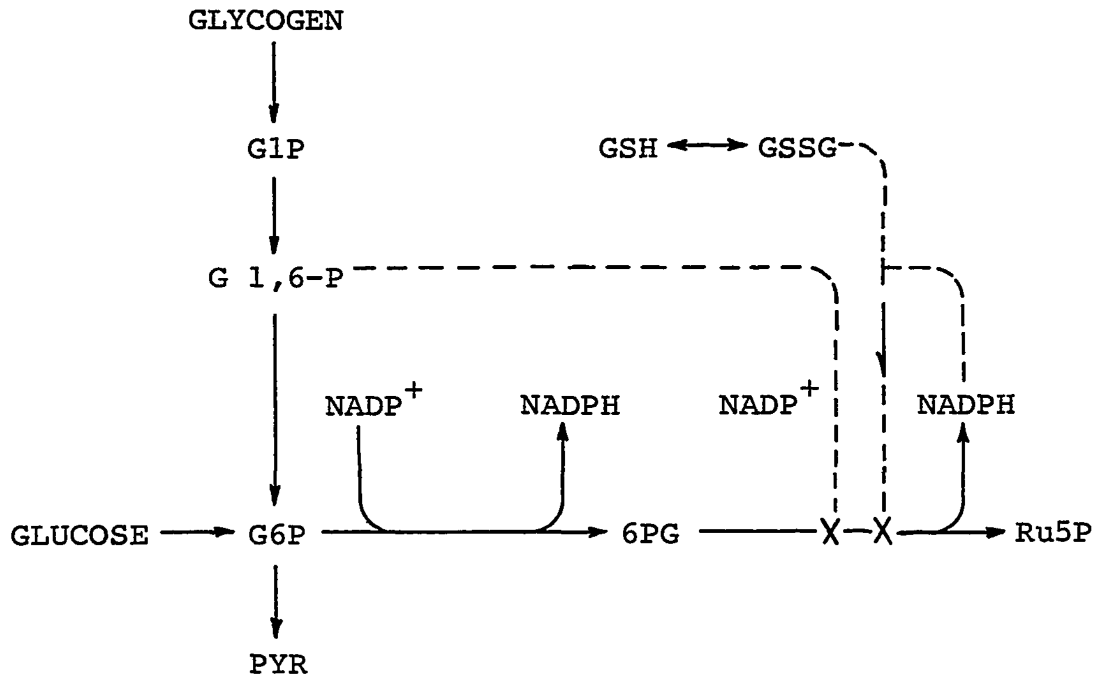

Fig. 12. Control of 6-phosphogluconate dehydrgeonase. Inhibition is indicated by a cross, and activation by a thick arrow.

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on the enzyme, and determinations of the concentrations of the various substrates, cofactors and modulators, that have been discussed, under the different physiological conditions of hibernation and nonhibernation. Even so, it is valid to propose a model of 6-phosphogluconate dehydrogenase regulation based on what we have found in this study, supplemented with information obtained from studies on other related animals. This model appears to be sufficient to allow the animal to meet the metabolic requirements involving the HMP pathway without having to resort to the costly process of producing different forms of the enzyme adapted to different environmental conditions.

In conclusion, the Arctic ground squirrel is an example of an organism efficiently adapting to large changes in environmental conditions without having to resort to extreme and likely costly measures. Rather than producing isoenzymes, a much touted and sometimes useful means of adaptation, we find it using changing environmental conditions to control enzyme activity to adapt to these same conditions.

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LITERATURE CITED

Atkinson, D. E. 1968. The energy charge of the adenylate pool as a regulatory parameter: Interaction with feedback modifiers. Biochem. 7:4030-4034.

Baquer, N. Z., M. Cascales, B. C. Teo and P. McLean. 1973. The activity of the pentose phosphate pathway in isolated liver cells. B. B. R. C. 52(1):263-269.

Behrisch, H. W. 1973. Molecular mechanisms of temperature adaptation in arctic ectotherms and heterotherms. in: Effects of Temperature on Ectothermic Organisms. (ed: W. Wieser). Springer-Verlag, Berlin.

Behrisch, H. W. 1974. Temperature and the regulation of enzyme activity in the hibernator. Isoenzymes of liver pyruvate kinase from the hibernating and nonhibernating Arctic ground squirrel. Can. J. Biochem. 52(10):894-902.

Behrisch, H. W. 1975. On the subunit structure of seasonal isoenzymes of fructose 1,6-diphosphate from muscle of the Alaskan king crab. Comp. Biochem. Phys. 51B:317-321.

Behrisch, H. W. 1978. Metabolic economy at the biochemical level: The hibernator. in: Strategies in Cold. (ed: L. Wang and J. Hudson). Academic Press, New York.

Behrisch, H. W. and C. E. Johnson. 1974. Regulatory properties of pyruvate kinase from liver of the summer active ground squirrel. Can. J. Biochem. 52(6):547-559.

Behrisch, H. W. and J. A. Percy. 1974. Temperature and the regulation of enzyme activity in homeo- and heterothermic tissues of arctic marine mammals: Some regulatory properties of 6-phosphogluconate dehydrogenase from adipose tissue of the spotted seal. Comp. Biochem. Phys. 47B:437-443.

Beitner, R. and J. Nordenberg. 1979. Inhibition of 6-phosphogluconate dehydrogenase (decarboxylating) by glucose 1,6-bisphosphate. Biochem. Biophys. Acta. 583(2):266-269.

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Bergmeyer, H. U. 1974. Methods of Enzymatic Analysis, 2nd Edition, vol. 2. Academic Press, New York.

Cleland, W. W. 1967. The statistical analysis of enzyme kinetic data. Advan. Enzymol. 29:1-32.

Dao, M. L., J. J. Watson, R. Delaney and B. Connor Johnson. 1979. Purification of a new high activity form of glucose 6-phosphate dehydrogenase from rat liver and the effect of enzyme inactivation on its immunochemical reactivity. J. B. C. 254(19): 9441-9447.

Denyes, A. and J. D. Carter. 1961. Utilization of acetate-1-C^{14} by hepatic tissues from cold-exposed and hibernating hamsters. Am. J. Phys. 200:1043-1046.

Dyson, J. E. D. and R. E. D'Orazio. 1971. 6-phosphogluconate dehydrogenase from sheep liver: Inhibition of the catalytic activity by fructose-1,6-diphosphate. B. B. R. C. 43(1):183-188.

Dyson, J. E. D. and R. E. D'Orazio. 1973. Sheep liver 6-phosphogluconate dehydrogenase inhibition by nucleoside phosphates and by other metabolic intermediates. J. B. C. 248(15):5428-5435.

Dyson, J. E. D., R. E. D'Orazio and W. H. Hanson. 1973. Sheep liver 6-phosphogluconate dehydrogenase: Isolation procedure and effect of pH, ionic strength and metal ions on the kinetic parameters. Arch. Biochem. Biophys. 154:623-635.

Eggleston, L. V. and H. A. Krebs. 1974. Regulation of the pentose phosphate cycle. Biochem. J. 138:425-435.

Fang, M. and J. M. Lowenstein. 1967. Citrate and the conversion of carbohydrate into fat. Biochem. J. 105:803-811.

Galster, W. and P. R. Morrison. 1975. Gluconeogenesis in Arctic ground squirrels between periods of hibernation. Am. J. Phys. 228(1):325-330.

Greenbaum, A. L., K. A. Gumaa and P. McLean. 1971. The distribution of hepatic metabolites and the control of the pathways of carbohydrate metabolism in animals of different dietary and hormonal status. Arch. Biochem. Biophys. 143:617-663.

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Hannon, J. P. 1960. Intermediary glucose metabolism in the cold acclimatized rat. Arctic Aeromedical Laboratory. Technical Report 60-150:1-25.

Hannon, J. P. and D. A. Vaughan. 1961. Initial stages of intermediary glucose catabolism in the hibernator and nonhibernator. Am. J. Phys. 201:217-223.

Hock, J. R. 1960. Seasonal variation in physiologic function of Arctic ground squirrels and black bears. in: Mammalian Hibernation. (ed: C. P. Lyman and A. R. Dawe). Bull. Mus. Comp. Zool. (Harvard) 124:155-171.

Holten, D., D. Proscal and H. L. Chiang. 1976. Regulation of pentose phosphate pathway dehydrogenases by NADP/NADPH ratios. B. B. R. C. 68(2):436-441.

Johnson, B. C. and H. F. Sassoon. 1967. Studies on the induction of liver glucose 6-phosphate dehydrogenase in the rat. Advan. Enz. Reg. 5:93-106.

Krebs, H. A. and L. V. Eggleston. 1974. The regulation of the pentose phosphate cycle in rat liver. Advan. Enz. Reg. 12:421-434.

Layne, E. 1957. Spectrophotometric and turbididmetric methods for measuring proteins. in: Methods in Enzymology. (ed: S. P. Colowick and N. O. Kaplan). vol. III:447-453. Academic Press Inc., New York.

Lehninger, A. L. 1975. Biochemistry, 2nd Edition: The Molecular Basis of Cell Structure and Function. Worth Publishers Inc., New York.

Lyman, C. P. and P. O. Chatfield. 1955. Physiology of hibernation in mammals. Phys. Rev. 35:403-425.

Mack, D. O., J. J. Watson and B. C. Johnson. 1975. Regulation of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the meal-fed rat. J. Nutr. 105(6):714-717.

Malan, A., H. Arens and A. Waechter. 1973. Pulmonary respiration and acid-base state in hibernating marmots and hamsters. Resp. Phys. 17:45-61.

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Malan, A., F. Daull and J. L. Rodeau. 1976. Relations entre les états acide-base extra-et intracellulaire dans l'acidose de l'hibernation. J. de Phys. (Paris) 72:105A.

McLean, P. and K. A. Gumaa. 1969. University of Miami Symposia on Control Mechanisms in Intermediary Metabolism. Miami, Florida.

Morrison, P. 1960. Some interrelations between weight and hibernation function. Bull. of the Mus. of Comp. Zool. 124:75-91.

Newsholme, E. A. and C. Start. 1973. Regulation in Metabolism. John Wiley and Sons Inc., New York.

Numa, S., W. M. Bortz and F. Lyman. 1964. Regulation of fatty acid synthesis at the acetyl-CoA carboxylation step. Advan. Enz. Reg. 3:407-423.

Olsson, S-O. R. 1972. Dehydrogenases in the heart, liver, white and brown fat. in: Seasonal Variations in the Physiology and Biochemistry of the European Hedgehog including Comparisons with Nonhibernators, Guinea Pig and Man. (ed: B. W. Johnson). Acta Phys. Scand., Stockholm.

Proscal, D. and D. Holten. 1972. Purification and properties of rat liver 6-phosphogluconate dehydrogenase. Activity at normal *in vivo* concentrations of coenzyme. Biochem. 11(7):1310-1314.

Reeves, R. B., B. J. Howell and H. Rahn. 1977. Protons, proteins, temperature: Publication in acid-base physiology. Dept. of Phys., SUNY-Buffalo.

Robert, M. and I. Gray. 1972a. Enzymatic mechanisms during temperature acclimation of the blue crab. I. Oxygen consumption and activity of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Comp. Biochem. Phys. 42B(3):377-387.

Robert, M. and I. Gray. 1972b. Enzymatic mechanisms during temperature acclimation of the blue crab. II. Kinetic and thermodynamic studies of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Comp. Biochem. Phys. 42B(3):389-402.

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Rodriguez-Segade, S., A. Carri'on and M. Freire. 1979. Isolation and purification of a regulating cofactor of the pentose-phosphate pathway. B. B. R. C. 89(1):148-154.

Rudack, D., E. M. Gozukara and D. Holten. 1971. The effect of dietary carbohydrate and fat on the synthesis of rat liver 6-phosphogluconate dehydrogenase. Biochem. Biophys. Acta. 252:305-313.

Sapag-Hagar, M., R. Lagunas and A. Sols. 1973. Apparent unbalance between the activities of 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase in rat liver. B. B. R. C. 50(1):179-185.

Schimke, R. T. and D. Doyle. 1970. Control of enzyme level in animal tissues. Ann. Rev. Biochem. 39:929-977.

Segel, I. H. 1976. Biochemical Calculations, 2nd Edition. John Wiley and Sons Inc., New York.

Siperstein, M. D. and V. M. Fagen. 1958. Studies on the relationship between glucose and oxidation and intermediary metabolism. J. Clin. Invest. 37: 1196-1201.

Sokoloff, L. I. 1960. Metabolism of the central nervous system *in vivo*. in: Handbook of Physiology-Neurophysiology. (ed: J. Field) vol. 3, chapter 77:1843-1864. Williams and Wilkins, Baltimore.

Steiner, D. F. and J. King. 1964. Induced synthesis of hepatic UDP-glucose, glycogen, glucotransferase after insulin administration of alloxan-diabetic rats. J. B. C. 239(5):1292-1298.

Tashima, L. S., S. J. Adelstein and C. P. Lyman. 1970. Radioglucose utilization by active, hibernating and arousing ground squirrels. Am. J. Phys. 218(1):303-309.

Tepperman, J. and H. M. Tepperman. 1958a. Effects of antecedent food intake pattern on hepatic lipogenesis. Am. J. Phys. 193:55-64.

Tepperman, H. M. and J. Tepperman. 1958b. The hexosemonophosphate shunt and adaptive hyperlipogenesis. Diabetes 7(6):478-485.

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Tepperman, J. and H. M. Tepperman. 1961. Metabolism of glucose-1-C^{14} and glucose-6-C^{14} by liver slices of refed rats. Am. J. Phys. 200:1069-1073.

Toews, M. L., M. I. Kanji and W. R. Carper. 1976. 6-phosphogluconate dehydrogenase: Purification and kinetics. J. B. C. 251(22):7127-7131.

Veech, R. L. 1968. Measurement of respiratory metabolites in animal tissue. D. Phil. Thesis, Univ. of Oxford.

Veech, R. L., L. V. Eggleston and H. A. Krebs. 1969. The redox state of free nicotinamide adenine dinucleotide phosphate in the cytoplasm of rat liver. Biochem. J. 115:609-619.

Villet, R. H. and K. Dalziel. 1972. Studies of 6-phosphogluconate dehydrogenase from sheep liver. 2. Kinetics of the oxidative-decarboxylation reaction, coenzyme binding and analyses for metals. Eur. J. Biochem. 27:251-258.

Whitten, B. K. and G. J. Klain. 1969. NADP specific dehydrogenases and hepatic lipogenesis in the hibernator. Comp. Biochem. Phys. 29:1099-1104.

Wilber, C. G. and X. J. Musacchia. 1950. Fat metabolism in the Arctic ground squirrel. J. of Mammalogy 31(3):304-309.

Yamauchi, T., J. J. Stegeman and E. Goldberg. 1975. The effects of starvation and temperature acclimation of pentose phosphate pathway dehydrogenases in brook trout liver. Arch. Biochem. Biophys. 167(1):13-20.

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