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TEMPERATURE AND THE REGULATION OF ENZYME ACTIVITY IN POIKILOTHERMS: REGULATORY PROPERTIES OF FISH FRUCTOSE-1,6-DIPHOSPHATASE

by

HANS WERNER BEHRISCH

B.Sc., University of British Columbia, 1964

M.A., Oregon State University, 1966

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Department of Zoology

The University of British Columbia
Vancouver 8, Canada

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ABSTRACT

The regulatory properties of fructose 1,6-diphosphatase (FDPase) from liver of rainbow trout (Salmo gairdneri), South American lungfish (Lepidosiren paradoxa) and migrating pink salmon (Oncorhynchus gorbuscha) were examined over the physiological temperature ranges of the organisms.

In rainbow trout saturation curves for substrate (fructose 1,6-diphosphate), and a cofactor (Mg^{2+}) are sigmoidal, and the evidence suggests cooperative interaction between the binding sites for these ligands. The affinity of the trout enzyme is approximately 50-to 100-fold higher for Mn^{2+} than for Mg^{2+} and the Mn^{2+} saturation curve is hyperbolic. The enzyme is inhibited by Ca^{2+} and Zn^{2+} and this inhibition appears to be competitive with respect to cofactor. The trout FDPase has an alkaline pH optimum and high pH values enhance FDPase affinity for cofactor. Low concentrations of 5'AMP inhibit the rainbow trout FDPase and the enzyme-AMP interaction is sensitive to temperature; thus, K_i for AMP at 25° is approximately 30-fold higher than at 0°. Furthermore, increasing concentrations of the cofactors tend to reduce and/or reverse the AMP inhibition and it is suggested that these ions may play a role in regulating the efficiency of AMP as an inhibitor of FDPase activity.

Similar to the trout FDPase, substrate and cofactor saturation curves for the lungifsh FDPase are sigmoid and

Hill plots of the data suggest homotropic interaction between their respective binding sites. Affinity of the lungfish enzyme for substrate increases markedly at low temperature, with the result that at physiological concentrations of substrate, the velocity of the reaction is essentially independent of temperature. In addition, increasing pH values stimulate enzyme activity and increase affinity of the lungfish FDPase for cofactor. The lungfish enzyme is inhibited by AMP, as is the trout FDPase, but affinity for the allosteric inhibitor is reduced and this FDPase-AMP interaction is independent of temperature. It is suggested that the decreased sensitivity to AMP is adaptive in view of the aestivating habit of the South American lungfish, under which condition the animal would be starved for long periods of time and a low energy charge (high concentrations of AMP) would probably exist in the liver cell.

The regulatory properties of FDPase from migrating salmon were investigated. The calculated energy charge of the liver cell from these fish is very low (0.464) in keeping with the extended starvation and high rates of muscular and biosynthetic activity in these organisms. As in the trout and lungfish enzymes, affinity of salmon FDPase for substrate increases with a decrease in temperature. As a result at physiological concentrations of substrate enzyme activity is independent of temperature. Arrhenius plots of the saturation kinetics are complex and suggest an interconversion of one or more forms of the enzyme. Subsequent

examination of the enzyme by sucrose density gradient centrifugation at different temperatures indicates that such thermally-dependent changes in conformation of the protein do occur, a finding which also offers a basis for the observed temperature-dependent changes in K_m for substrate. The affinity of salmon FDPase for its allosteric inhibitor, AMP, is lower than in other FDPases and this enzyme-AMP interaction is largely insensitive to temperature. The functional significance of this reduced sensitivity to AMP is that it allows normal, indeed high, FDPase activity during conditions of low energy charge. These findings suggest mechanisms for the maintenance of high rates of gluconeogenesis in salmon during spawning migration.

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CHAPTER 1

INTRODUCTION

"Regulation is, of course, an essential condition of life. Metabolic regulation is not a late development somehow grafted on to pre-existing metabolic sequences; the sequences and their regulation necessarily evolved together, with mutation and selection acting in most cases on the same molecules in the development of catalytic efficiency and of regulatory response." (Atkinson, 1968).

For the first half of this century, biochemistry went through a stage of descriptive and largely empirical work. This classical approach began to undergo a major reversal after most of the basic framework of metabolism had become known. Researchers in the area were now faced with the problem of interpreting an immense body of empirical, often disjointed pieces of information. Biochemists started to draw heavily upon the theories of evolution and natural selection; as a consequence, the themes of adaptive significance and selective advantage, previously almost alien concepts in biochemistry, are now central to many areas of research.

One of the first examples of an analysis of biochemistry in functional terms is contained in the Herter lectures by Krebs (1954 a,b,c). Since then, this trend toward an interpretation of metabolic chemistry against an evolutionary background has been exemplified by the work of Atkinson (1965,

1966, 1968) and Stadtman (1968) in which current theories of enzyme regulation are argued in teleological terms. In this context the factors which govern the rate and direction of metabolic flow are considered in terms of the 'demands' and 'supply' of the cell. The application of this functionalistic philosophy has, and continues to contribute very significantly to the recognition of integrated reaction sequences as metabolic units with special function and hence selective value.

In these terms, just as the individual reaction has meaning only in light of the sequence in which it occurs, the sequence is useful only in the light of its integration into the overall metabolism of the organism. The primary function of regulatory enzymes is to effect this integration.

Modulation of enzyme activity by metabolites was first recognized in connection with regulation of biosynthetic pathways and it was early considered that such control might be at the molecular level (Adelberg and Umbarger, 1953; Koch _et al_. 1952; Abelson _et al_. 1952; Gots and Chu, 1952; Love and Gots, 1955). Indeed, as early as 1950, Gots had proposed a molecular inhibitor of purine synthesis in _E. coli_. After the initial discoveries of end-product inhibition of two biosynthetic pathways (Umbarger, 1956; Yates and Pardee, 1956), a number of important findings were made in the field. Umbarger (1956) and Yates and Pardee (1956) found that some enzymes, known to be subject to feedback inhibition,

catalyzed physiologically irreversible reactions. In fact, irreversibility of the controlled enzyme was early recognized as being important in the regulation of a biosynthetic pathway (Umbarger, 1956), and Yates and Pardee (1956) considered the advantages of having control placed at an irreversible step in a biosynthetic sequence. Control at a later point in the sequence would be an inefficient arrangement, since intermediates would be synthesized whether they were needed or not, thus resulting in a wasting of energy and metabolites. Subsequently it was found that asparate transcarbamylase (Gerhart and Pardee, 1962) and threonine dehydrase (Changeux, 1961) exhibited a decrease to their respective inhibitors without loss of their catalytic activity. These and similar findings led to the proposal of the existence of separate sites for substrate and modulator on the enzyme molecule (Gerhart and Pardee, 1962; Monod and Jacob, 1962). Although these sites were thought to be separate they interacted in that they influenced the binding of their respective ligands. Changeux (1961, 1962) suggested a model enzyme with two sites, each able to bind substrate or regulator, in varying combinations. Monod and Jacob (1962) then suggested that the regulator need not be a steric analogue of the substrate by referring to such interaction as 'allosteric', a term which has since been loosely employed in a variety of contexts. The proposal that binding of the regulator molecule leads to a change in the conformational

state of the enzyme has served as the basis for most subsequent considerations of enzyme regulation. Umbarger and Brown (1957) and Halpern and Umbarger (1959) reported two cases in which a given reaction is catalyzed by two enzymes, one of which had a degradative function and the other a biosynthetic function. Stadtman et al. (1961) reported the first example of parallel enzymes that both participate in biosynthesis, both of which are subject to inhibition by the end-product. Findings from these and other workers prompted Monod, Changeux and Jacob (1963) to postulate the existence of entirely separate, rather than overlapping, substrate and inhibitor sites. Since most enzymes have unique substrates and specific modulators, they suggested that this type of regulation was a basic characteristic of living systems, and that it could have arisen only through mutation and selection; that is, it was adaptive.

The concept of allosterism is thus one of the cornerstones to current theory in enzyme regulation. An extension of this concept is the idea of cooperative interaction between two or more ligand binding sites per molecule of enzyme; thus the initial binding of one molecule of ligand effects a conformational change in the enzyme, the result of which is an enhancement of binding of further ligand molecules at other sites (Koshland, 1963). This behaviour yields a sigmoid substrate saturation curve, a common feature of regulatory enzymes. One of the characteristics of enzymes displaying

sigmoid saturation curves is that small changes in substrate concentration can lead to large changes in activity. By the same token, small changes in affinity for substrate can cause large variations in reaction velocity. Most regulatory molecules exert their influence by modulating affinity of enzyme for substrate, thus creating a very efficient method of control. A far-reaching implication is that physiological concentrations of substrate are generally far below those required for maximal activity, since only at low concentrations would affinity for substrate be significant in determining reaction rate. Finally, if control of regulatory enzymes is to be considered adaptive, the activity and therefore the contributions of their respective pathways must be a reflection of the demands of the cell or organism for their products.

Ecological considerations: Poikilotherms are exposed to a great variety of environmental stresses and adapt to these stresses in many different ways. Lungfish will aestivate during the dry season and have been known to survive up to several years in this condition. Similar adaptations are known to occur in a number of molluscs (Martin, 1961). The maintenance of metabolism and its control over this period is synonymous with survival. Desert fishes experience marked changes in temperature and the ionic environment within relatively short periods of time, and will remain active all the time (Bishai, 1960). In the intertidal situation, changes in temperature and salinity may be quite pronounced. In tidal

flats, for instance, the temperature may range from about 7 - 8° to as high as 40° (Dr. Wallace Heath, personal communication). Very large salinity changes (from 0.5 to 28 parts per thousand within an hour period) can occur during tidal changes. Allied to these variations is the availability of oxygen to the organisms (Oysters, clams, nereid worms) in this area. Many tidal flats or marshes have a very reducing environment and thus are effective oxygen sinks; as a result oxygen availability is reduced. Many poikilotherms can adapt to this type of situation and can survive for considerable lengths of time in an absolutely oxygen-free world. During the northern winter, many ponds and lakes are completely covered by ice and the oxygen supply is completely cut off. Blažka (1958) found that the crucian carp (Carassius carassius) can survive for longer than five months with no oxygen at temperatures around 5°. The fish do not accumulate lactic acid or fatty acids, regularly eliminate CO_2, and accumulate newly synthesized fat. Blažka also found similar adaptations in developing fish eggs. Active fishes, such as the salmonids, do not have this capacity for anaerobiosis and tend to accumulate acid metabolites in the tissues and contract oxygen debts during periods of oxygen deficiency (Black et al. 1961). Compounding this are the actions of hormones, which are produced in response to a variety of stimuli. Thus, starvation will lead to increased production of cortico-steroids which enhance gluconeogenesis (Exton and Park, 1965;

Barboriak, 1967), but which at high concentrations may also play a role in mineral metabolism (Gorbman and Bern, 1964). The changes thus incurred in the animal's ion profile may in turn affect the enzymes of intermediary metabolism (see Bygrave, 1967), and the response of these enzymes to other environmental factors, such as temperature. In amphibians, metamorphosis is thought to be the result of increased thyroid activity (Gorbman and Bern, 1964). As a result of this metamorphosis, the entire ecology of the animal changes, and with it the food habits and numerous aspects of intermediary metabolism. Furthermore, thyroid hormone causes changes in the intermediary metabolism of fish (Hochachka, 1962) and is known to accelerate gluconeogenesis from pyruvate, lactate and glycerol (Freedland and Krebs, 1967; Joseph and Subrahmanyam, 1968).

Perhaps one of the animals in which the most spectacular (and highly visible) adaptations occur, is the Pacific salmon. These fish do not feed during their long migration upstream to the spawning beds, so that energy is supplied through the metabolism of fats and amino acids (Idler _et al_. 1959; Chang and Idler, 1960). The extended starvation is complicated by a high degree of muscular and biosynthetic activity, a condition which would define a low energy charge in the cell which would in turn inhibit many biosynthetic pathways (see Atkinson, 1968). A complicating effect may be seen as the result of hormonal changes which are known to occur in these animals during spawning migration (Idler _et al_. 1959).

These hormonal changes presumably affect numerous aspects of metabolism, either directly or indirectly. In addition, the change from an environment of high ionic strength and constant temperature to one of low ionic strength and varying temperatures would also place different demands on the organism and might be expected to affect certain aspects of metabolism. Similar changes are known to occur in the European eel Anguilla during its spawning run to the sea (Hoar, 1965).

In summary, poikilotherms respond to the vagaries of the environment in a variety of ways. In some such as the rainbow trout there is an acclimatory response and the animal remains active throughout much of its thermal range. Others, such as the South American lungfish (Lepidosiren paradoxa) aestivate during certain parts of the year and so avoid having to adapt to changes in the environment; still others conform (see Precht, 1968). Despite these varied problems, molecular control mechanisms in poikilotherms are essentially unknown.

Although a large literature is devoted to metabolic adaptations of poikilotherms to a variable environment (Read, 1964a,b, 1967; Roberts, 1966; Mutchmor, 1967; Jankowsky, 1968; Precht, 1968), most previous studies have been largely descriptive. Questions of molecular mechanism: (1) How are poikilothermic enzymes adapted for function under conditions of changing temperature, ionic strength, pressure, oxygen

availability, etc., (2) Are the regulatory characteristics of poikilothermic enzymes unique, (3) What enzyme characteristics appear to be most sensitive to natural selection during adaptation to unique environments? - have been largely ignored.

Statement of the Problem

Of all the environmental variables that have been considered with respect to adaptation of metabolism, the one that has received the most attention is temperature and a number of reviews on the subject exist (for example, see Hochachka, 1967; Hochachka and Fry, in press; Hochachka and Somero, 1968). The basic problems of metabolic control in poikilotherms arise from the fact that most of the aspects of enzyme regulation are directly affected by temperature. In mammalian and bacterial systems, large and often differential effects of temperature on enzyme-modulator and enzyme-substrate interactions can occur (Ingraham and Malløe, 1967; Helmreich and Cori, 1964; Taketa and Pogell, 1965; Iwatsuki and Okazaki, 1967b). Intuitively, it appeared that the types of changes observed for homeothermic enzymes, as well as their magnitude, would be incompatible with survival in poikilothermic systems. For example, if the magnitude of the change in enzyme-modulator interaction observed for rat liver fructose 1,6-diphosphatase (FDPase) (Taketa and Pogell, 1965) should occur in poikilothermic systems, then one would expect that gluconeogenesis would be completely "switched off" at low temperatures, whereas, in salmonid fishes at least, it

appears to be favoured at low temperatures (Hochachka, 1967).

The resynthesis of glucose from other sources is essential for the maintenance of certain types of cells and as a supply for the immediate energy demands of the organism. Because the maintenance of normal concentrations of glucose is synonymous with survival, and since one of the earliest effects of temperature on regulatory properties of enzymes was made on the control enzyme of the gluconeogenic pathway (Taketa and Pogell, 1965), it was decided to examine the kinetic and regulatory properties of fructose 1,6-diphosphatase in relation to temperature adaptation.

Literature survey: The glycolytic sequence is characterized by a number of reactions that are essentially irreversible (e.g. the phosphorylation of fructose-6-phosphate by phosphofructokinase (PFK), and the hydrolysis of phosphoenolpyruvate to yield pyruvate). If gluconeogenesis is to occur, these steps must be circumvened, and this takes place through a number of steps which are themselves irreversible. Because of their undirectional nature, these steps make logical metabolic valves, and selection appears to have favoured the development of strict control of enzymes at such junctures in their various pathways (see Yates and Pardee, 1956; Umbarger, 1956).

Over the past few years a considerable body of information on regulatory enzymes in glycolysis and gluconeogenesis has accumulated. From a consideration of the scheme

of these pathways as outlined by Utter et al. (1964), Krebs (1964), Caputto et al. (1967) and Scrutton and Utter (1968), regulatory steps may be identified at points which (1) initiate reactions of major substrates, such as amino acids, pyruvate, lactate, glycerol and fructose, (2) relate to the dispersal of shared intermediates to more than one pathway, (3) are specific to either pathway. For example, while the glycolytic and gluconeogenic pathways share a number of enzymes, other enzymes have a specialized function for one pathway only. Thus, pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase and glucose-6-phosphatase function only in gluconeogenesis, while hexokinase, phosphofructokinase and pyruvate kinase function only in glycolysis. In a scheme for the control of both rate and direction of carbon flow, Weber (1963) predicted the strict regulation of such enzymes in intermediary metabolism.

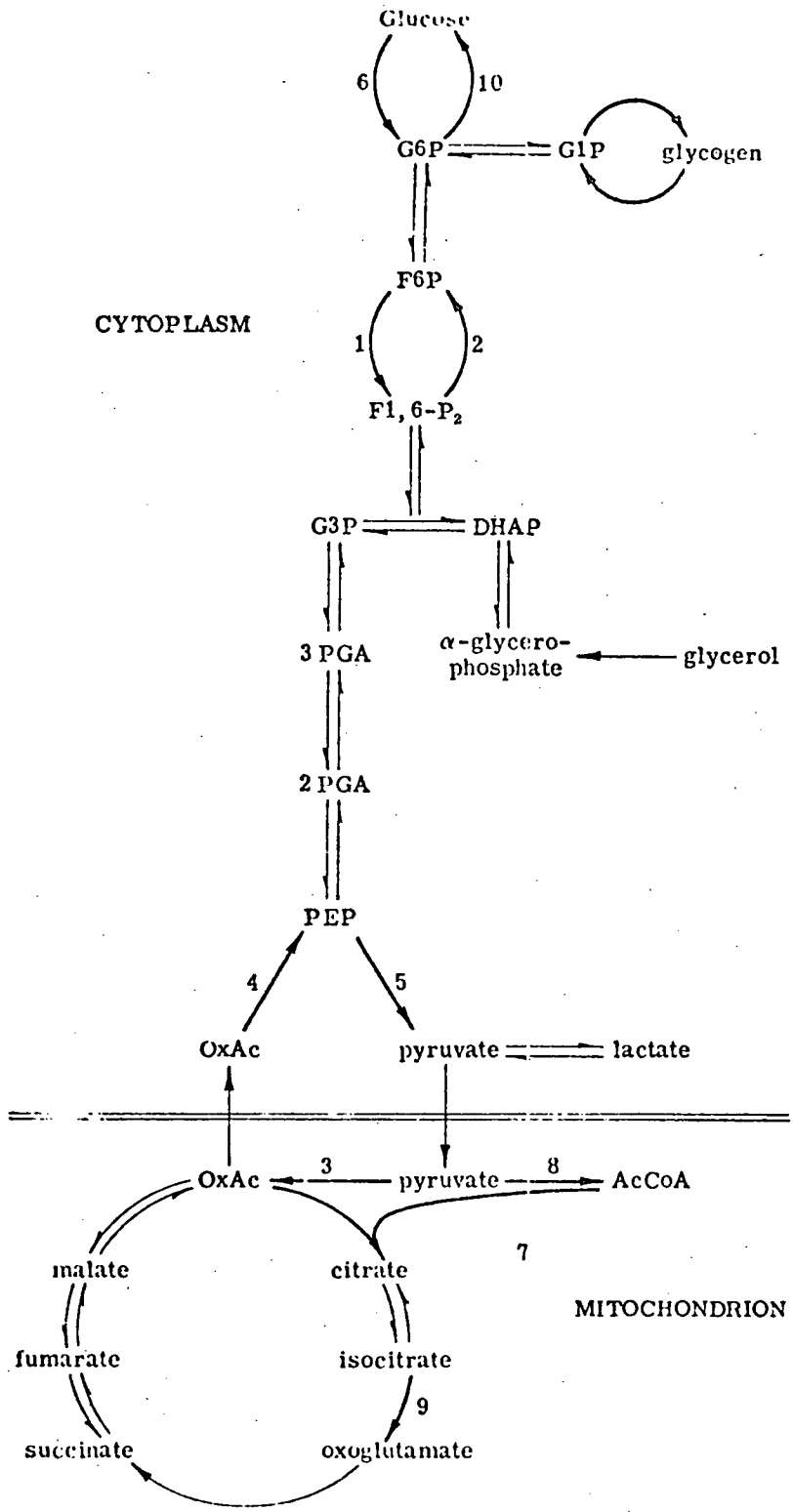
A number of workers (e.g. Weber et al. 1961; Niemeyer et al. 1963; Krebs and Eggleston, 1965; Henning et al. 1966) have reported changes in the activities of a number of the above enzymes during dietary or hormonal treatments in mammals. It has been suggested that if changes in enzyme activities which are associated with changes in the net flow of carbon were specific for regulatory enzymes, it would then be possible to identify regulatory enzymes. However, such changes appear to be non-specific. Thus, Schimke (1962) showed that nearly all the enzymes of the urea cycle behaved similarly in

response to variations in the rate of urea synthesis in starving rats. In addition, Fitch and Chaikoff (1960) showed that the extent and patterns of hepatic enzyme activity, when normal rats were fed high glucose or fructose diets and compared with rats on normal diets, suggested that most of the enzymes that were involved with portions of pathways with high activity were increased. Similarly Weber et al. (1961) demonstrated that cortisone administration to adrenalectomized rats produced not only increases in FDPase and G-6-Pase activities (enzymes with low activity) but also in phosphohexoseisomerase (a "high-activity" enzyme common to both glycolysis and gluconeogenesis). It thus appears that investigations on enzyme adaptations are of limited value toward the positive identification of control points in metabolism. In this connection, the significance of the high increases in the activities of enzymes other than the pacemakers was examined by Krebs (1963). If the enzyme activities are very high relative to the amounts of substrate available then any substrate, as it becomes available, will react very rapidly and the steady state concentrations of the intermediates will be very close to the equilibrium state (Buecher et al. 1962; Hess, 1962). The implication of this is that the free energy changes occurring in the course of the intermediate steps are minimal and that any losses of free energy are reduced to negligible quantities. Thus the high capacity of certain enzymes may be understood as a useful arrangement in that it in fact constitutes economy of

energy instead of a wasting. Furthermore, it makes possible an understanding of why the ratios of enzyme activites are in many cases very constant; why not only the rate-limiting steps but also the other steps of a reaction sequence increase in activity when certain stresses make greater demands (for further discussion see Krebs, 1963).

It is now largely accepted that the following enzymes of glycolysis and gluconeogenesis catalyze non-equilibrium reactions: hexokinase, glucokinase, PFK, pyruvate kinase, pyruvate carboxylase, PEP carboxykinase, FDPase and G-6-Pase (Krebs, 1963). This is consistent with the finding that the enzymes G-6-Pase, FDPase, PEP carboxykinase and pyruvate carboyxlase are found in greatest quantities in the gluconeogenic tissues (kidney cortex and liver), and are considered necessary to circumvene the energy barriers of glycolysis (Newsholme and Gevers, 1967). The pathways leading from various precursors to glucose are summarized in Fig. 1.1. Energy barriers that prevent the reversal of glycolysis are present at (1) the direct conversion of pyruvate to PEP, (2) the transfer of phosphate from FDP to ADP, and (3) the transfer of phosphate from G-6-P to ADP. These barriers are circumvened by special reactions mediated by phosphoenolpyruvate carboxykinase (PEPCK), fructose 1, 6-diphosphatase (FDPase), and glucose-6-phosphatase (G-6-Pase), respectively. The energy barrier between fructose 1,6-diphosphate (FDP) and fructose-6-phosphate (F-6-P) is overcome

Figure 1.1 Some regulatory enzymes involved in metabolic control of glycolysis and gluconeogenesis. The enzymes are as follows:
(1) phosphofructokinase (PFK);
(2) fructose 1,6-diphosphatase (FDPase);
(3) pyruvate carboxylase
(4) phosphoenolpyruvate carboxykinase (PEPCK);
(5) pyruvate kinase (PK);
(6) hexokinase (or glucokinase);
(7) citrate synthase;
(8) pyruvate cehydrogenase;
(9) NAD-isocitrate dehydrogenase;
(10) glucose-6-phosphatase.
(From Newsholme and Gevers, 1967).


Glucose
6
10
G6P
G1P
glycogen
F6P
CYTOPLASM
1
2
F1, 6-P2
G3P
DHAP
3 PGA
α-glycero-phosphate
glycerol
2 PGA
PEP
4
5
OxAc
pyruvate
lactate
OxAc
3
pyruvate
8
AcCoA
7
malate
citrate
MITOCHONDRION
fumarate
isocitrate
9
succinate
oxoglutamate

by a specific phosphatase, which occurs mainly in the two gluconeogenic tissues, liver and kidney (Krebs, 1963). The enzyme is a "low capacity" enzyme (Krebs, 1963), which would indicate that it limits the rate of the overall sequence. Activity of the enzyme increases during glucose-low diets or cortisone administration to the animal (Mokrasch et al. 1956; Weber et al. 1961; Weber et al. 1964). Furthermore, the enzyme is inhibited by AMP (Taketa and Pogell, 1963, 1965; Underwood and Newsholme, 1965), and Gevers and Krebs (1966) have shown that varying concentrations of the adenylates at this step can effect changes in glucose synthesis in homogenates of pigeon liver. Schimassek (1963) and Schimassek and Mitzkat (1963) treated perfused livers of rats with glucagon and measured the concentrations of metabolites in the tissue. They reported increased uptake of lactate and a fall in the steady state contents of pyruvate and lactate. The authors also reported a decrease in the content of FDP and a rise in hexose-6-phosphate concentrations, as well as some evidence for increased gluconeogenesis from lactate. Since FDP is a substrate of an enzyme that catalyzes a non-equilibrium reaction, this finding further suggests that FDPase is a regulatory enzyme in gluconeogenesis. Underwood and Newsholme (1967) investigated the rates of glucose synthesis from triose phosphate precursors in rat kidney cortex slices and also obtained evidence for the regulatory nature of FDPase in gluconeogenesis.

In 1943 Gomori first described a phosphatase which he found in liver and kidney of human, dog, guinea pig, rabbit and rat. The enzyme specifically hydrolyzed FDP to F-6-P and had an alkaline pH optimum around pH 9.0, a value considerably above the accepted physiological pH. The enzyme was first purified by McGilvery and Pogell (1954) who also found it to have a very alkaline pH optimum. Mokrasch and McGilvery (1956) purified FDPase from rabbit liver and demonstrated that the enzyme had an absolute requirement for a cationic cofactor, which could be satisfied by either Mg^{2+} or Mn^{2+}; in addition Mokrasch and McGilvery found that excess substrate (FDP) brought about inhibition of the enzyme, a finding later confirmed by Krebs (1963) and Taketa and Pogell (1965).

The molecular weights of the FDPases from a wide number of organisms have been calculated, and the values range from 100,000 to 140,000. Rosen _et al_. (1966) have purified the enzyme from _Candida utilis_, and at pH 7.0 the enzyme sediments in sucrose density gradients with a molecular weight of about 100,000. When the same experiment was carried out at pH 4.0, a single somewhat broader peak was obtained with a molecular weight of about 40,000 to 50,000. These halves had approximately 10% the catalytic activity of the native enzyme, but affinity for substrate had not changed. A molecular weight of 137,000 has been reported for rabbit liver FDPase (Pontremoli _et al_. 1966); at low pH (pH 2.0) the protein dissociated into subunits

of molecular weight approximately 75,000. These subunits were inactive, but neutralization of the enzyme apparently resulted in a reassociation of the subunits and a return of catalytic activity. Experiments by Horecker et al. (1966) on N-terminal and C-terminal amino acids suggested that the enzyme may contain subunits which may be different, or which may themselves be composed of different smaller subunits. In this connection, Ellis et al. (1966) reported the dissociation of bovine liver FDPase into two pairs of subunits corresponding to molecular weights of 24,000 and 48,000, and Sia et al. (1969) report the presence of 2 pairs of subunits of molecular weight 29 - 31,000 and 35 - 39,000, respectively, for rabbit liver FDPase.

A series of experiments by Pontremoli et al. (1965a, b,c,) on dinitrophenylation of sulfhydryl groups led to the suggestion that activity of the enzyme was dependent on the state of ionization of one or more sulfhydryl groups. When the sulfhydryl groups are undissociated, the enzyme is inactive; titration of the sulfhydryl leads to the ionization of the group and a marked increase in activity. This result, the authors reason, could explain the finding that FDPase has a marked alkaline pH optimum, since raising the pH presumably leads to the dissociation of the sulfhydryl group and an increase in catalytic activity. This sulfhydryl group does not appear to be part of the active site itself, since dinitrophenylation does not lead to a blocking of enzymic activity. However, it appears that the group must

be located at an allosteric site, the ionization or modification of which leads to a change in conformation and hence activity of the enzyme. It was subsequently reported (Pontremoli *et al*. 1965d) that cysteine slightly stimulates activity at neutral pH, and that the titration of two sulfhydryl groups with iodoacetate or para-chloromercuribenzoate causes marked stimulation of activity.

FDPase has an absolute requirement for a bivalent cationic cofactor, which may be satisfied by either Mg^{2+} or Mn^{2+} (Gomori, 1943; Pontremoli *et al*. 1965a; Horecker *et al*. 1966). The pH optimum of FDPase has been shown to be dependent on the concentration and identity of cofactor. Thus, it has been reported that purified rabbit muscle FDPase in the presence of high concentrations of Mg^{2+} has an optimum around pH 7.5; when the Mg^{2+} level is reduced or Mn^{2+} substituted, the pH optimum shifts to more alkaline pH values. In this connection, Preiss *et al*. (1967) find that increasing concentrations of Mg^{2+} shift the pH optimum of spinach leaf FDPase to the neutral range. Most FDPases, although they are activated by both Mg^{2+} and Mn^{2+}, show maximum activity with one or the other. Thus the FDPase from the slime mold *Dictyostelium discoideum* shows three times as much activity in the presence of Mg^{2+} as in the presence of Mn^{2+} (Baumann and Wright, 1969).

There is conflicting evidence about the binding of substrate (FDP) by the enzyme. FDPase has a high affinity

for substrate (Km = 1-3 μM for rat liver FDPase, Taketa and Pogell, 1965), and most substrate saturation curves are hyperbolic, indicating little or no cooperative interaction between the FDP binding sites (Taketa and Pogell, 1965; Salas et al. 1964; Underwood and Newsholme, 1965a,b,c); Mendocino and Vasarhely, 1963; Fraenkel et al. 1966; Rosen et al. 1966). In contrast, for the spinach leaf FDPase, substrate saturation curves are sigmoidal in nature, and Hill plots of the results yield values of n between 1.3 and 1.8, indicating homotropic cooperation between the FDP binding sites (Preiss et al. 1967). Preiss and his coworkers also observed that the degree of interaction between the substrate binding sites was markedly dependent upon the concentration of Mg^{2+} present in the medium. In this connection, Pontremoli et al. (1968) using the technique of gel filtration, reported the existence of four FDP binding sites per molecule of enzyme; the interaction between sites appears to be dependent upon the pH of the medium. At pH 7.5 there is a cooperative effect between the FDP binding sites; when pH is raised to pH 9.2, this effect is not detectable. However, Sarngadharan et al. (1969) using the same techniques, were unable to observe FDP site-site interaction, although their finding of 4 FDP sites agrees with that of Pontremoli and coworkers. The question is as yet unresolved at this writing.

Fructose diphosphatase catalyses an irreversible reaction, in order to circumvene the energy barrier imposed

by the PFK reaction. Since one of the substrate of the PFK reaction is ATP, and the products of the reaction are ADP and FDP, it is reasoned that the coincident full operation of both enzymes would result in the net hydrolysis of ATP, and thus cause a short-circuit in metabolism (Horecker et al. 1966). For this reason, it is held that both enzymes must be tightly controlled, and a large body of information on the subject exists. There is some evidence, however, that some cycling of intermediates through the FDPase-PFK cycle does occur (Gevers and Krebs, 1966; Newsholme and Underwood, 1966; Underwood and Newsholme, 1967). This question has been considered in depth by Newsholme and Gevers (1967) who discuss the advantages of having apparently "wasteful" cycles present in metabolism. In the case of PFK and FDPase, the activity of the former enzyme is modified in a complex manner by a number of regulatory metabolites, whereas FDPase is modified by only AMP, and possibly FDP. If both enzymes are simultaneously, but not fully, active, the regulators of PFK may affect the activities of both pathways. In this way direction and net flow of carbon could depend on a number of factors: e.g., changes in concentrations of citrate, AMP, ATP, and FDP; on the other hand, if only FDPase is active, the rate of gluconeogenesis would be controlled solely (at this step) by changes in AMP concentration. It is thus reasoned that the loss of energy during cycling of hexose phosphates at this level may be compensated for by the increased versatility of control

(Newsholme and Gevers, 1967). Evidence exists that such cycles are present also at the phosphoenolpyruvate-pyruvate interconversion level (Gevers, 1967; see also Scrutton and Utter, 1968).

As a control enzyme, FDPase is inhibited by 5'-AMP. The inhibition is allosteric and early studies suggested the existence of two interacting AMP-binding sites (Taketa and Pogell, 1965). Binding of AMP was not competitive with respect to the substrate (Taketa and Pogell, 1965), although it appears that the binding of AMP is enhanced in the presence of substrate (Wanatabe _et al_. 1968; Pontremoli _et al_. 1968b; Sarngadharan et al. 1969). There are four AMP binding sites per molecule of FDPase and there is evidence of homotropic cooperation between the AMP binding sites (Sarngadharan _et al_. 1969). It was first pointed out by Taketa and Pogell (1965) that the interaction between FDPase and AMP is remarkably dependent on temperature. Thus, at 46° the K_i of AMP in rat liver FDPase is approximately 100 times its value at 2°. Similarly Lowry _et al_. (1964) found an increase in the affinity of phosphorylase a from rabbit muscle for its activator, 5'-AMP. This kind of temperature effect has also been observed in deoxythymidine kinase from _E. coli_, in which a decrease in temperature effects a rise in affinity for substrate (Iwatsuki and Okazaki, 1967). Furthermore, a rise in temperature from 0 to 37° results in a normalization of the usually sigmoid saturation curve for the substrate, ATP. Such changes in enzyme-substrate affinity undoubtedly are

linked to fundamental conformational changes in the enzyme (Iwatsuki and Okazaki, 1967).

If AMP modulation were the major controlling mechanism, and if the FDPase-AMP interaction were as temperature-sensitive as in homeothermic organisms, then efficiency of metabolic control (at this level) in a poikilotherm would be unusually sensitive to changes in environmental temperature. This implies that FDPase from coldwater fishes is perhaps less sensitive to negative control by AMP at low temperatures or that mechanisms are available for the activation of FDPase and/or reversal of AMP inhibition (see Pogell _et al_. 1968). For these reasons, it was decided to examine the role of ions in addition to those of substrate and AMP in the regulation of FDPase. Since the kinetic and regulatory properties of a number of intermediary enzymes are known to be highly temperature-sensitive, it was also decided to investigate the thermal characteristics of the FDPases from three fishes with different thermal histories.

Rainbow trout were chosen because these organisms in Nature encounter fairly wide variations in temperature, and thermal adjustments of salmonids are fairly well documented (see Hochachka, 1967; Dean, 1969; Baldwin and Hochachka, in preparation. The South American lungfish has evolved in what is essentially a thermally stable environment. However, during the dry season, when the animal may become stranded in isolated pools, the lungfish probably comes under thermal stress, made all the more acute by the air-breathing habit

which brings the fish to the warmest surface layers. In periods of drought, the lungfish will aestivate and can survive in this condition for long periods of time. This extended starvation would probably result in a reduced energy charge which would tend to inhibit the FDPase reaction and gluconeogenesis. However, the maintenance of normal levels of blood glucose is an absolute requirement for survival, and Janssens (1964, 1965) reports gluconeogenesis from amino acid precursors. This suggests that other important control mechanisms are perhaps operative. Migrating salmon were chosen because these animals do not feed during their journey to the spawning beds. The state of starvation is compounded by high muscular and biosynthetic activities, a condition which could result in a low energy charge, which in turn would inhibit gluconeogenesis. However, gluconeogenesis from amino acid precursors is known to be maintained in these organisms (Chang and Idler, 1960). In addition, the fish migrate from an environment of high ionic strength and constant temperature to one of low ionic strength and variable temperatures. The temperature changes may be particularly pronounced in the summer months, when the level of the stream may be down and flow rate can change abruptly.

The basic concern of this study is to determine the manner in which one regulatory enzyme, fructose 1,6-diphosphatase, is adapted to these various physiological and ecological conditions.

CHAPTER 2

MATERIALS AND METHODS

I. Rainbow Trout

(a) Experimental methods: Hatchery-raised (Sun Valley Trout Farms, Coquitlam, B.C.) rainbow trout were kept at 10° and were fed *ad libidum*. Liver samples were excised immediately after death and kept in a deep freeze until use. Liver homogenates were prepared in 3 parts of 0.01 M Tris-HCl, pH 7.4, in a VirTis homogenizer at 1 - 2°. The homogenates were spun at about 30,000 g for 30 minutes at 4° and the pellets discarded. The supernatant was brought to 15% saturation with solid $(NH_4)_2SO_4$ and stirred for at least 3 hours at 4°C. The suspension was then centrifuged as above, the pellets discarded, and the supernatant brought to 55% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and then dissolved in a minimal volume of 0.01 M Tris-HCl buffer, pH 7.4. The enzyme solution was extensively dialysed against 0.01 M Tris-HCl (pH 7.4) at 4° before use in the assays. This procedure separated FDPase from non-specific alkaline phosphatase which was assayed with para-nitrophenylphosphate (Sigma Chemical So., St. Louis, Mo., U.S.A.) as substrate. The reaction medium contained in 2.0 ml. of 0.05 M Tris-HCl buffer, various concentrations of FDP, 5'-AMP (Sigma Chemical Co., St. Louis,

Mo., U.S.A.), Mg^{2+} or Mn^{2+} ions and Ca^{2+} or Zn^{2+} ions. Care was taken to maintain pH constant at all temperatures, by correcting for the effect of temperature on the ionization of Tris. The reaction was initiated by adding the FDPase preparation and was assayed by measuring P_i release. The P_i was determined by the Fiske and Subbarow (1925) method by using the Technicon Autoanalyzer (Technicon Science Center, Tarrytown, New York, U.S.A.). Unless otherwise specified, all kinetic experiments were carried out at 15° and pH 7.4. Enzyme activity was linear with time for at least 30 minutes under optimum conditions, and was proportional to enzyme concentration.

(b) Sucrose density gradient centrifugation: The method of sucrose density centrifugation was developed by Martin and Ames (1961) for the study of physical characteristics of proteins. The method involves a small volume of dilute protein solution (about 5 mg./ml.) on a sucrose density gradient prepared in a celluloid centrifuge tube. Centrifugation is then carried out in the swinging bucket rotor (SW 39) in the preparative ultracentrifuge (Spinco Model L). After completion of centrifugation, a hole is bored in the bottom of the centrifuge tube and fractions are collected and analyzed.

For these experiments a linear gradient of 5 percent sucrose (w/v) versus 20 percent sucrose (w/v) was prepared. The sucrose solutions were prepared in 0.01 M Tris-HCl, pH

7.4. The prepared gradients, of 4.6 ml. in volume, were chilled in an ice bucket or were stored at 4° in the cold room before use. The gradients were used within 2 hours after preparation. The enzyme solution was carefully applied to the top of the gradient in order to prevent the formation of bubbles and to assure that the protein sample formed a sharp interface with the gradient. Centrifugation was carried out at 39,000 rpm in a pre-cooled rotor, and in a pre-cooled centrifuge chamber, for 12 hours. The rotor was brought to a stop without the use of the brake in order to avoid disturbing the gradients. The tubes were punctured with a small needle and approximately 35 samples of 2 drops each were collected at 4°. Enzyme activity was assayed at 25°.

(c) Acrilamide gel electrophoresis: FDPase preparations from cold (2°) and warm (18°) acclimated trout were examined by standard acrylamide gel electrophoresis at 4°C, using a 4 percent stacking gel, 7 percent separating gel and tris-glycine tank buffer, pH 8.3. Gels were run for 90 minutes at 3 ma. per tube and then cut into sections about 2 mm. in thickness. The slices were then assayed FDPase for activity as above.

II. Lungfish

(a) Experimental animals: The fish used in this study, *Lepidosiren paradoxa*, inhabits the non-turbulent waters of swamps and flooded rain forests in South America. The fish

is an obligate air breather, surfacing at regular intervals for air. During May and June the Amazon drainage is at high water and much of the rain forest is in flood. At this time the surface temperature of the water is about 27° and shows little or no daily thermal fluctuations. Although there is no thermocline in the open river systems, thermoclines may develop in the still waters of the rain forest (H. Ungermeyer, personal communication), and small (1 - 2°) thermal gradients have been seen in the surface waters here. In general, however, conditions are stable at this time of year and do not impose a thermal stress on the animal. Later, when the waters recede from the forest, isolated pools are often formed in which the temperature rises. In some of these H. Ungermeyer (personal communication) has recorded temperatures as high as 38°. Any fish stranded in such pools could come under rather severe thermal stress, made all the more acute by the air-breathing habit, which brings the fish to the warmest surface layers. In periods of extreme drought the lungfish can go into prolonged aestivation.

The South American lungfish, Lepidosiren, was obtained from the Museum Goeldi, Belém, Brazil, and was captured in the swamp waters of the area. The animals were in captivity for at least several weeks before these experiments and were starved during this time.

(b) Experimental methods: Lungfish liver homogenates

were prepared in three parts of 0.01 M Tris-HCl buffer, pH 7.4, in a VirTis homogenizer at 1 - 2°C. The homogenates were spun at about 30,000 g for 30 minutes at 4° and the pellets were discarded. The supernatant was brought to 30 percent saturation with solid $(NH_4)_2SO_4$ and stirred for at least 3 hours at 4 - 5°. The suspension was then centrifuged as above and the pellet discarded. The solution was extensively dialysed against 0.01 M Tris-HCl buffer, pH 7.4, at 4°. The preparation contained no detectable non-specific phosphatase, which was assayed with para-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as substrate. Lungfish FDPase activity was assayed as above.

III. Migrating Salmon

(a) Experimental animals: Pink salmon (*Oncorhynchus gorbuscha* Walbaum) that had not yet spawned were seined on the Bella Coola River in British Columbia. The temperature of the water was about 10 - 13°C. Liver samples were excised immediately after sacrifice, and kept at -25° until use.

(b) Experimental methods: Enzyme solutions were prepared in the same manner as from rainbow trout liver.

Total nucelotide levels in the liver cell from spawning salmon were determined by use of ATP and ADP/AMP assay kits (C.F. Boehringer & Soehne, Mannheim, Germany).

These assays are not specific for the adenine nucleotides; the other nucleotides react quantitatively with the assays (Bergmeyer, 1965). However, the relative concentrations of all the tri-, di-, and mononucleotides may be taken to reflect the relative proportions of adenylates and thus the energy charge of the cell. A pooled sample of 11 livers was homogenized in 3.5% perchloric acid at 4°C. The homogenate was spun at 5,000 g for 10 minutes at 4°, the precipitate discarded, and the supernatent used in the nucleotide determinations. Energy charge of the liver cells was calculated using the equation of Atkinson and Walton (1967).

CHAPTER 3

TEMPERATURE AND THE REGULATION OF ENZYME ACTIVITY IN POIKILOTHERMS: PROPERTEIS OF RAINBOW-TROUT FRUCTOSE DIPHOSPHATASE

INTRODUCTION

The enzyme FDPase in the presence of a bivalent cation, catalyses the conversion of FDP into fructose 6-phosphate and P_i. The requirement for close regulation of FDPase activity in gluconeogenic tissues is generally recognized, since the simultaneous operation of both FDPase and phosphofructokinase within a single cell would lead to hydrolysis of ATP and thus to a short-circuit in metabolism (Horecker, Pontremoli, Rosen & Rosen, 1966). A number of studies suggest that the concomitant inhibition of FDPase and activation of phosphofructokinase by AMP can prevent the simultaneous function of these two enzymes (Underwood & Newsholme, 1965a,b,c; Newsholme & Gevers, 1967). This postulate is supported by studies of liver homogenates insofar as adenylate modulation at this point affects the net direction of carbohydrate metabolism (Gevers & Krebs, 1966). An overlooked feature of FDPase control is a remarkable temperature-dependence of the FDPase-AMP interaction. Thus, at 46°, the K_i of AMP for the rat liver enzyme is about 100 times its value at 2° (Taketa & Pogell, 1965).

If AMP modulation were the major controlling mechanism, efficiency of control in a poikilotherm would be unusually sensitive to environmental temperature changes, and glucose production would be essentially "turned off" at low temperatures, whereas, in salmonid fishes at least, it appears to be favoured at low temperatures (Hochachka, 1967).

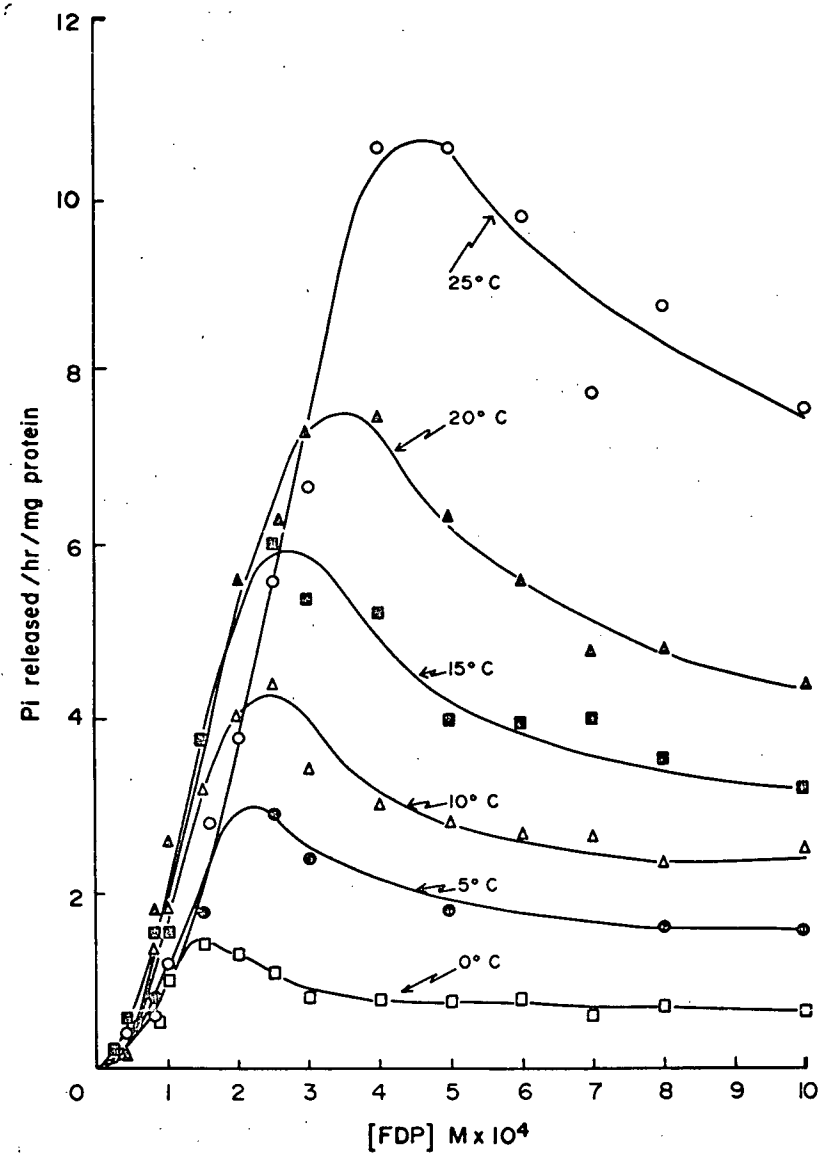
This implies that FDPase from salmonid fishes is perhaps less sensitive to AMP control at low temperature or that other regulatory mechanisms are available. We therefore examined the role of cations in addition to those of substrate and AMP in the regulation of rainbow-trout liver FDPase. We chose rainbow trout because these organisms in Nature encounter fairly wide variations in temperature, and thermal adjustments of salmonids are fairly well documented (Hochachka, 1967).

RESULTS

Rainbow-trout liver FDPase, unlike most other types of FDPase examined (Horecker et al. 1966; Pontremoli, Luppis, Traniello, Wood & Horecker, 1965b), has a low affinity for substrate and a sigmoidal substrate-saturation curve (Fig.3.1). Hill plots of the results give slopes of about 2 from 5° to 25°, with 5 mM-Mg^{2+} in the reaction medium. These values may be taken, with reservation, to represent the degree of interaction between sites that bind the substrate, FDP, and agree with those for the spinach leaf enzyme (Preiss, Biggs & Greenberg, 1967). The slope of the Hill plot rises sharply at low temperature; thus at 0° n equals 4.6, indicating a great increase in FDP site - site interaction, with the result that the enzyme is saturated with substrate much more quickly. The value of n 4.6 that we find in the trout FDPase at 0° does not differ greatly from the number of binding sites calculated by Pontremoli, Grazi & Accorsi (1968) for rabbit liver FDPase.

Substrate inhibition of the trout enzyme is very apparent at pH 7.4 with either Mg^{2+} or Mn^{2+} as the cofactor, as has been reported for the rat liver enzyme (Taketa & Pogell, 1965). The enzyme is inhibited by about 50% at 1mM-FDP and optimum Mg^{2+} concentration and by about 25% at 1mM-FDP and optimum Mn^{2+} concentration (Fig.3.1), an effect first noted by Krebs (1963). Temperature apparently has little effect on the degree of inhibition by increased FDP, whether

Figure 3.1 Substrate saturation curves of rainbow trout FDPase at different temperatures at pH 7.40. Activity is expressed as 10^{-5} M P_i released/l./hr./mg. protein. The set of saturation curves on the left were carried out in the presence of 5×10^{-3} M Mg^{2+} and those on the right in the presence of 2×10^{-5} M Mn^{2+}.


Pi released /hr/mg protein
[FDP] M x 10^4
25° C
20° C
15° C
10° C
5° C
0° C

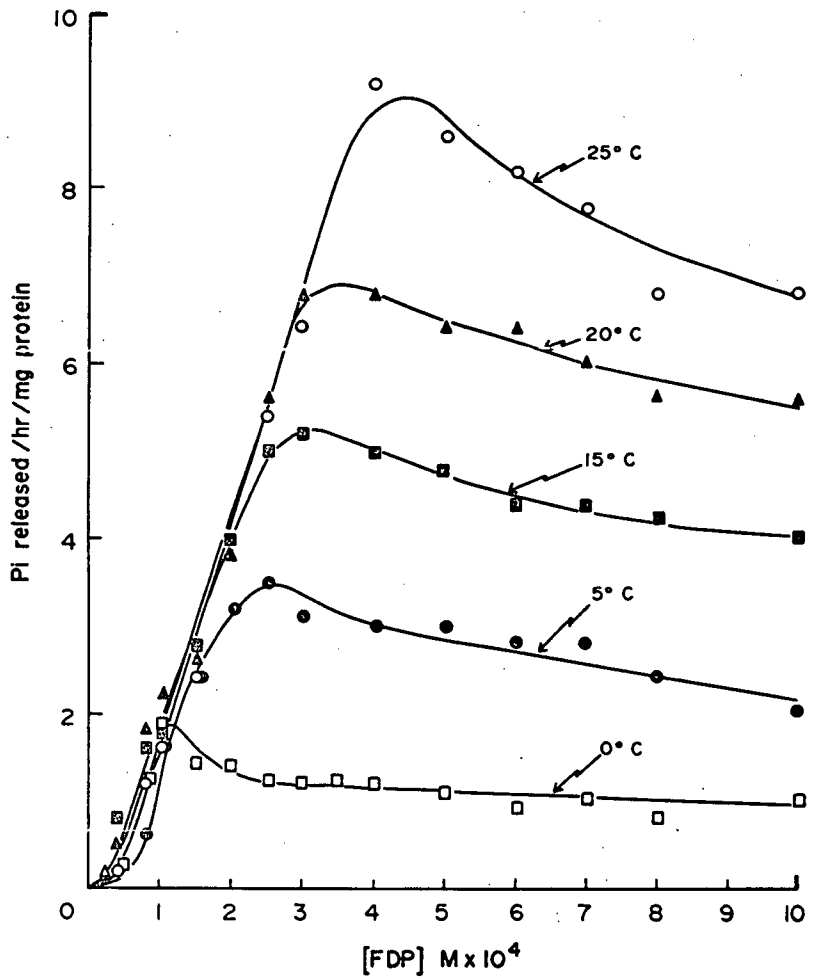

Pi released /hr/mg protein
[FDP] M x 10^4
25° C
20° C
15° C
5° C
0° C

Table 3.I Effect of temperature on affinity and values of n of substrate (FDP) of rainbow-trout FDPase at different pH values and concentrations of cofactor.

	pH 7.4						pH 9.0		
	5mM-Mg^{2+}			1mM-Mn^{2+}			1mM-Mn^{2+}		
Temp.	10^4K_m (M)	n	Probability of n being zero	10^4K_m (M)	n	Probability of n being zero	10^4K_m (M)	n	Probability of n being zero
0°	0.85	4.60	0.000	0.7	4.87	0.017			
5	1.2	2.30	0.001	1.1	4.17	0.003	1.1	4.74	0.000
10	1.2	2.59	0.016				1.2	4.56	0.000
15	1.3	1.71	0.000	1.5	2.39	0.012	1.0	2.73	0.000
20	1.5	2.54	0.000	1.7	2.05	0.002	1.0	2.16	0.000
25	2.4	1.72	0.000	2.1	1.81	0.000	1.4	1.78	0.000

Mn^{2+} or Mg^{2+} is the cofactor. There is thus a sharp peak of catalytic activity with a rapid decrease as substrate becomes more concentrated (Fig.3.1), suggesting that FDP serves both as a substrate and an effective modulator of the enzyme. We have no information on the mechanism for substrate inhibition; the results are consistent with those of Underwood & Newsholme (1965a) and Taketa & Pogell (1965), who suggested a competitive binding of two identically charged substrate molecules to the active site, resulting in an inactive complex.

Temperature does not appear to affect greatly the ability of the enzyme to bind FDP, although there is a fall in K_m from 5° to 0°, and a relatively large rise in K_m from 20° to 25°, when either Mg^{2+} or Mn^{2+} is the cofactor (Table 3.I). Substrate-saturation curves near the alkaline pH optimum (pH 9.0) showed almost the same results. The curves are markedly sigmoidal and there appears to be no substrate inhibition; there is a plateau of activity in each case (Fig.3.2). The sigmoidal saturation curves of the trout enzyme at pH 9.0 at all temperatures contrast with the findings of Pontremoli _et al_. (1968), who reported a loss of the co-operative effect between the four FDP-binding sites in the rabbit liver FDPase at pH 9.2, thus suggesting a hyperbolic substrate-saturation curve. As at pH 7.4, the slopes of Hill plots increase at low temperature, so that at pH 9.0 at 5° n is 4.74 and at 25° n is 1.78 (see Fig.3.3 and Table 3.I).

Figure 3.2 Substrate saturation curves of rainbow trout FDPase at pH 9.0 at a series of temperatures in the presence of 10^{-3} M Mn^{2+}. Activity is expressed as 10^{-5} M P_i releases/l./hr./mg. protein.

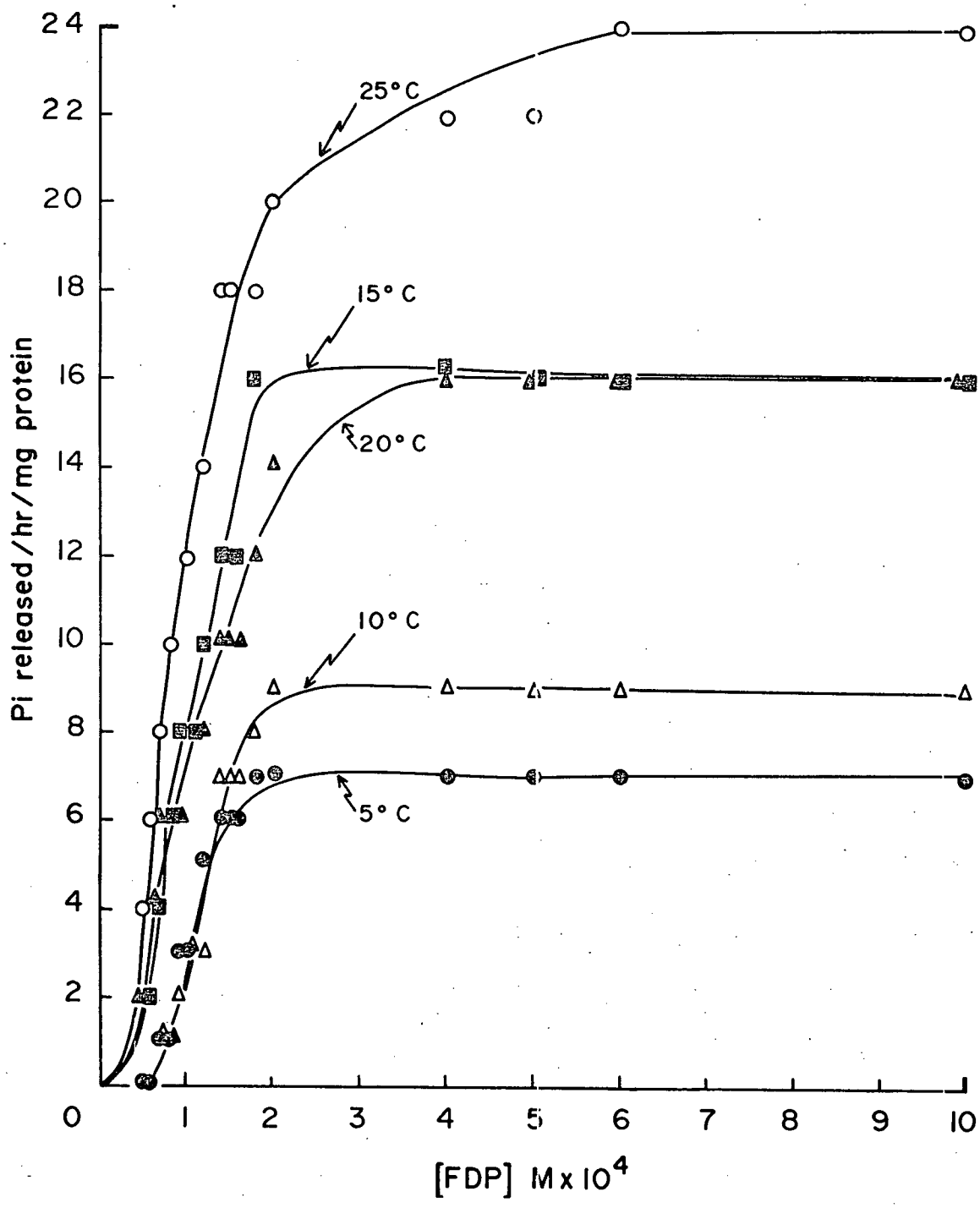

Pi released/hr/mg protein
24
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
[FDP] M x 10^4
25° C
15° C
20° C
10° C
5° C

Table 3.II Activation energies of rainbow-trout FDPase at several temperature ranges.

E_a was calculated from V_{opt}.

E_a (kcal./mole)

Temperature range	pH 7.40 5mM-Mg^{2+}	pH 7.40 20 µM-Mn^{2+}	pH 9.0 1mM-Mn^{2+}
0 - 5°	22.90	15.15	
5 - 10	11.98		7.89
5 - 15		6.26	
10 - 15	9.12		19.0
15 - 20	7.57	8.98	0
20 - 25	10.67	10.43	13.5

Figure 3.3 Hill plots for substrate saturation curves of rainbow trout FDPase at pH 9.0 at 5°, 10° and 15°. Assay conditions were as described in Fig. 2.

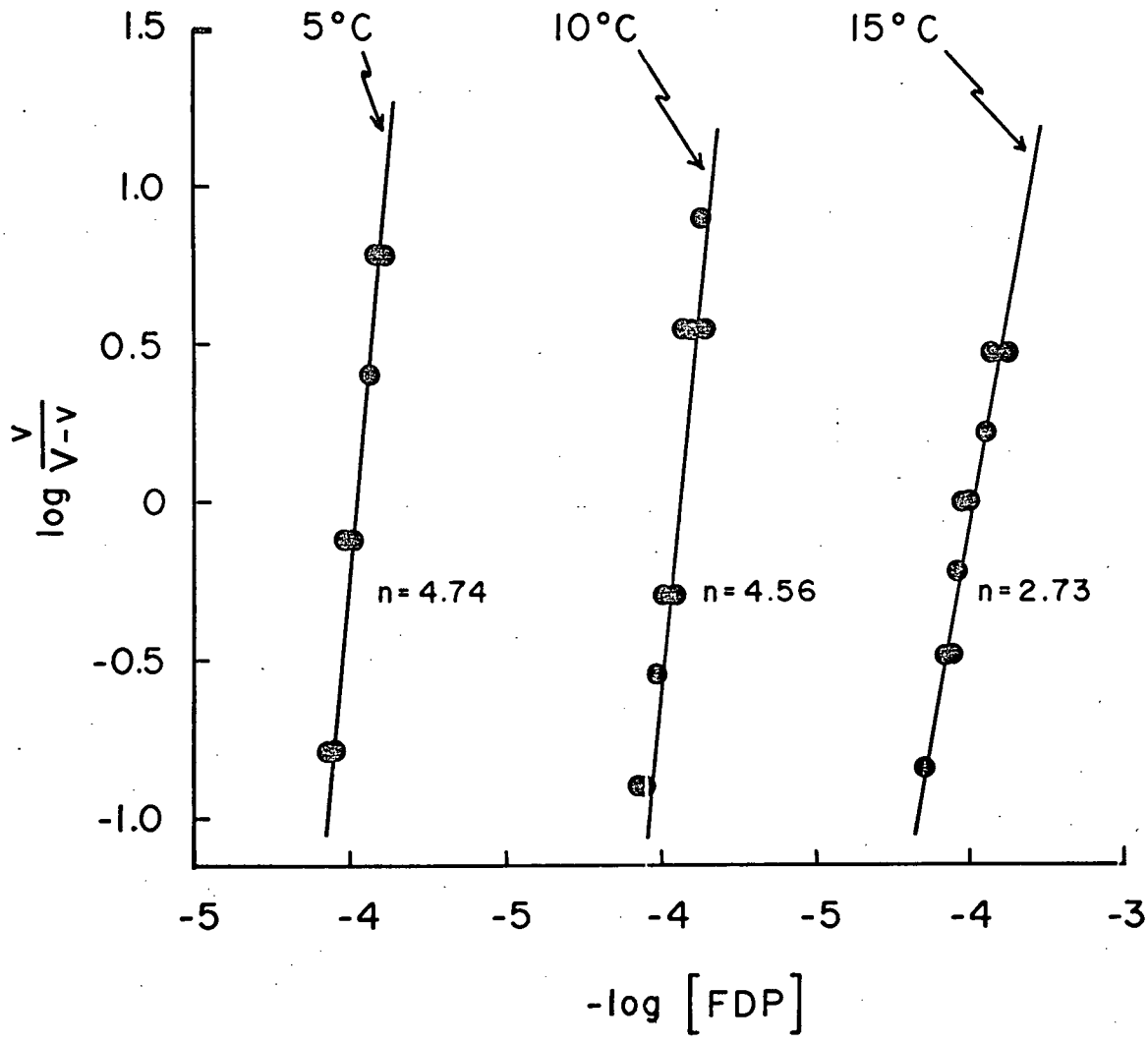

5°C
10°C
15°C
1.5
1.0
0.5
0
-0.5
-1.0
log v/(V-v)
n = 4.74
n = 4.56
n = 2.73
-5
-4
-5
-4
-5
-4
-3
-log [FDP]

Figure 3.4 Arrhenius plots for rainbow trout FDPase at pH 7.4 and pH 9.0. Values are calculated from reaction velocities under optimum conditions ($V_{opt.}$).

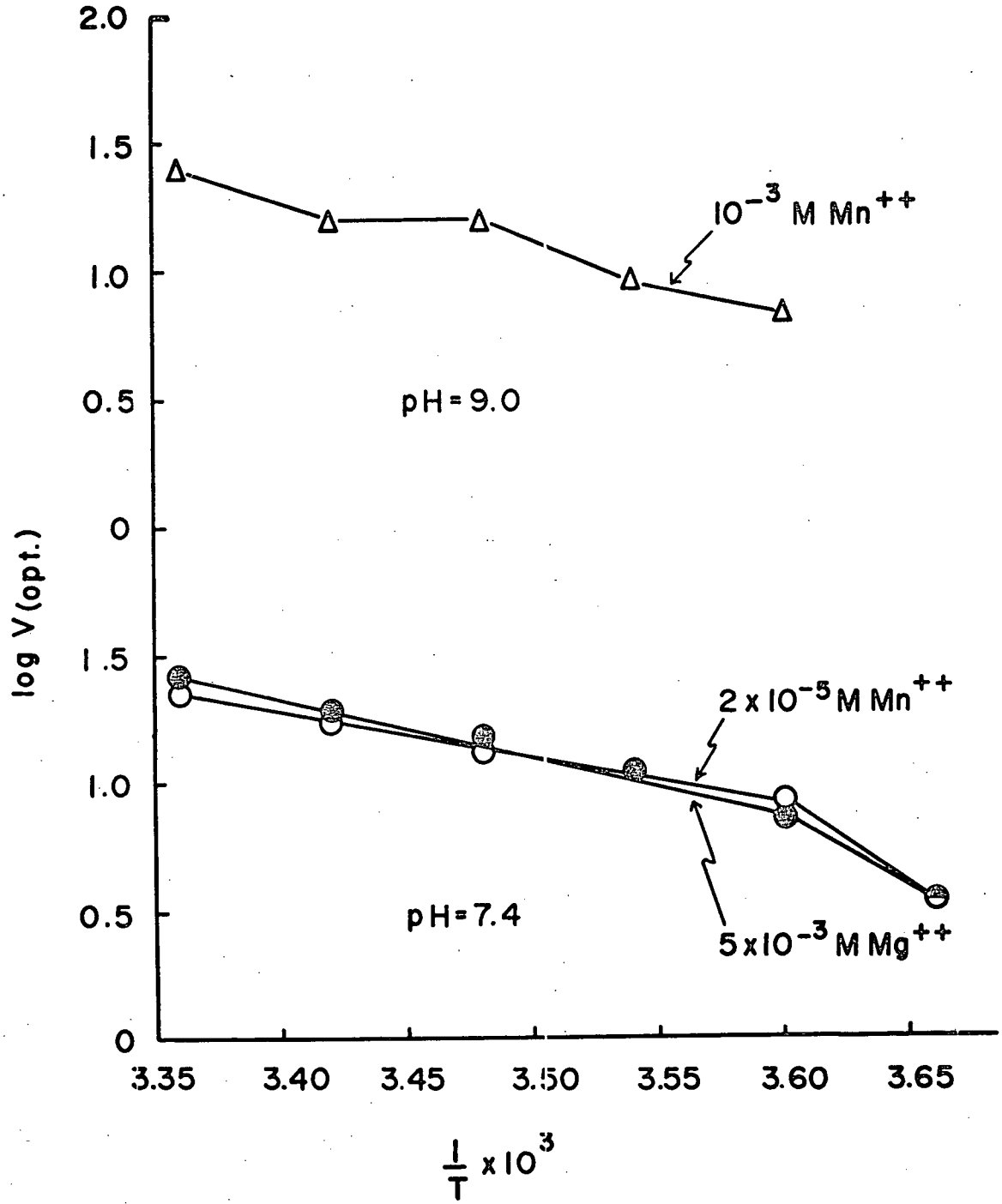
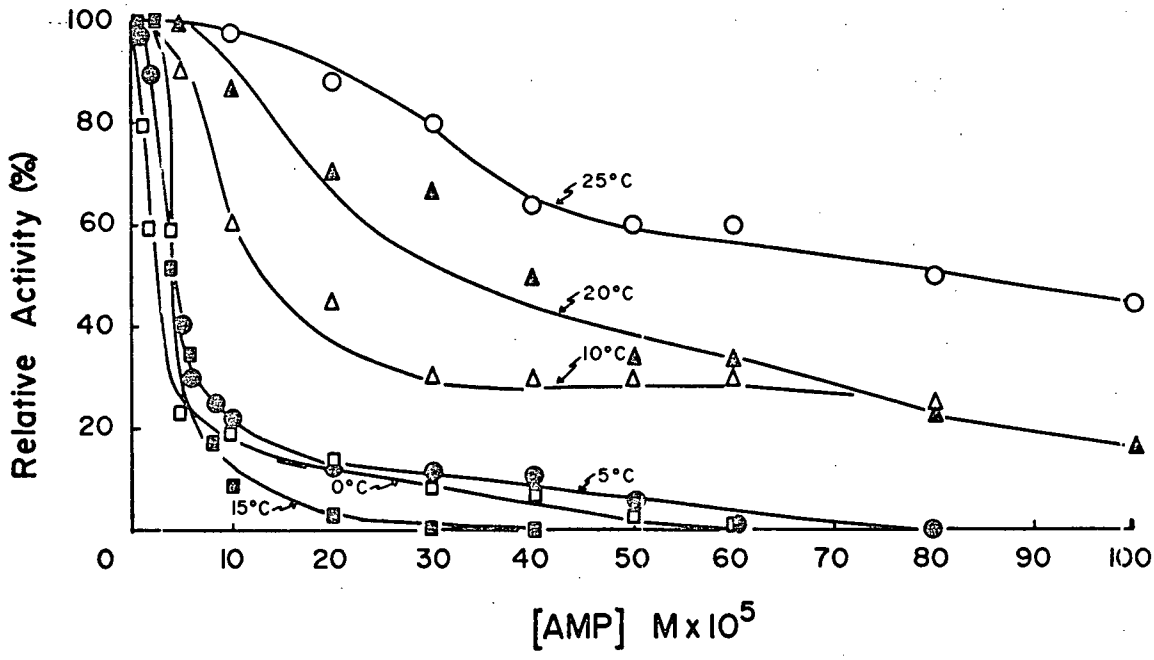

2.0
1.5
1.0
0.5
0
1.5
1.0
0.5
0
log V(opt.)
10^{-3} M Mn^{++}
pH = 9.0
2×10^{-5} M Mn^{++}
5×10^{-3} M Mg^{++}
pH = 7.4
3.35
3.40
3.45
3.50
3.55
3.60
3.65
$\frac{1}{T} \times 10^{3}$

Figure 3.5 Inhibition of rainbow trout FDPase by AMP. Assay conditions in the upper set of curves were pH 7.40, 3×10^{-4} M FDP and 10^{-3} M Mg^{2+} and varying temperatures. Assays in the lower group were carried out at pH 9.5, 15°C, and in the presence of 10^{-3} M FDP and various levels of Mg^{2+}.


Relative Activity (%)
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
100
25°C
20°C
10°C
5°C
0°C
15°C
[AMP] M x 10^5

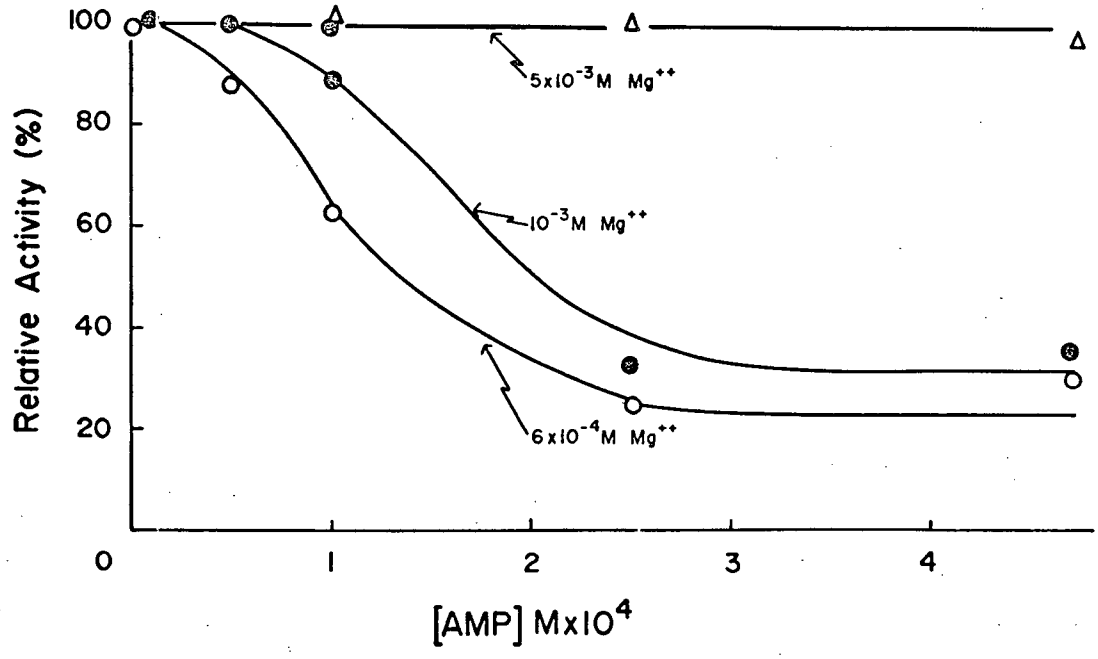

Relative Activity (%)
100
80
60
40
20
0
1
2
3
4
5x10^-3 M Mg^++
10^-3 M Mg^++
6x10^-4 M Mg^++
[AMP] M x 10^4

Table 3.III AMP inhibition of rainbow-trout FDPase at a series of temperatures: effect of temperature on values of n and K_i for AMP

Assays were carried out in 0.05 M-tris-HCl buffer, pH 7.40, in the presence of 0.3mM-FDP and 1mM-Mg^{2+}.

Temp.	$10^5 K_i$ for AMP (M)	n	Probability of n being zero
0°	2.5	1.10	0.000
5	4.5	1.49	0.000
10	13	0.97	0.000
15	4.5	2.39	0.000
20	33	1.52	0.000
25	80	1.65	0.000

The activation energy of the enzyme at 0 - 5° is approximately 1½-2 times that at higher temperatures (Table 3. II), and there is a break in the Arrhenius plot at 5° at pH 7.4 (Fig.3.4). At pH 9.0, there is a plateau between 15 and 20° in the Arrhenius plot, with a rise in activity at 20° (Fig.3.4). The enzyme thus appears to be temperature-insensitive between 15° and 20° at pH 9.0.

Inhibition by AMP: FDPase is strongly inhibited by AMP (Horecker *et al*. 1966; Taketa & Pogell, 1963, 1965; Underwood & Newsholme, 1965a). At pH 7.4 and at optimum concentrations of Mg^{2+} and FDP, the inhibition curve of FDPase is characteristically sigmoidal (Fig.3.5), and there appear to be two interacting sites that bind AMP per molecule of enzyme. This number of sites differs from the value of three estimated by Taketa & Pogell (1965) for the rat liver enzyme. Temperature has a strong effect on the affinity of the trout enzyme for AMP (Table 3.III. At 0°, K_i is 2.5 x 10^{-5}M, and at 25° the apparent K_i is 8 x 10^{-4}M, approximately a 30-fold increase. The above relationship between temperature and affinity of the enzyme for the inhibitor was also reported in rat liver FDPase (Taketa & Pogell, 1965), in which there is about 100-fold increase in K_i with a rise in temperature from 2° to 46°. These inhibition constants for the rainbow-trout enzyme agree closely with those for the K_i of the brook-trout liver enzyme (M. Bosomworth, H.W. Behrisch & P.W. Hochachka, unpublished work). Further

experiments with the lake-trout FDPase yielded confusing results; the enzyme from two separate preparatons was almost totally insensitive to AMP inhibition. Variability in AMP sensitivity has also been noted for the ox liver enzyme (W.L. Byrne, personal communication); it thus appears that there may be both sensitive and insensitive forms of the enzyme.

There is a conspicuous decrease in K_i for AMP in the rainbow-trout liver enzyme at 15° (Table 3.III) and a corresponding rise in the slope of the Hill plot at this temperature, a relationship not seen at the other temperatures, although K_i does change with temperature. In an attempt to find a possible explanation for this behaviour, we noted that K_a for Mg^{2+} was slightly higher at 15° than at any of the other temperatures tested, with the exception of 25°. The powerful inhibition of rainbow-trout liver FDPase at this temperature may be related to the relative decrease in affinity for the cofactor. Also, the nature of the enzyme-modulator or the enzyme-substrate complex, or both, may change with temperature, with the result that catalytic activity becomes more sensitive to AMP at 15°. Higher concentrations of Mg^{2+} or Mn^{2+} cause an increase in K_i and so protect against inhibition by AMP (Table 3.IV). In addition, Hill plots of these results demonstrate a definite fall in the value of n with increasing concentration of cofactor (Table 3.IV). It thus seems that higher concentrations of Mg^{2+} or Mn^{2+} cause a fall in AMP site-

Table 3.IV Effects of various concentrations of cofactor and pH values on values of n and K_i for AMP.

Assays were carried out at 15° in the presence of 1mM-FDP.

pH 7.4

Concn. of cofactor (mM)		$10^5 K_i$ for AMP (M)	n	Probability of n being zero
Mg^{2+}	0.6	5	1.58	0.008
	1.0	6.5	1.24	0.000
	5.0	9	1.08	0.000
Mn^{2+}	0.02	11	2.14	0.000
	0.01	13	0.906	0.002

pH 9.5

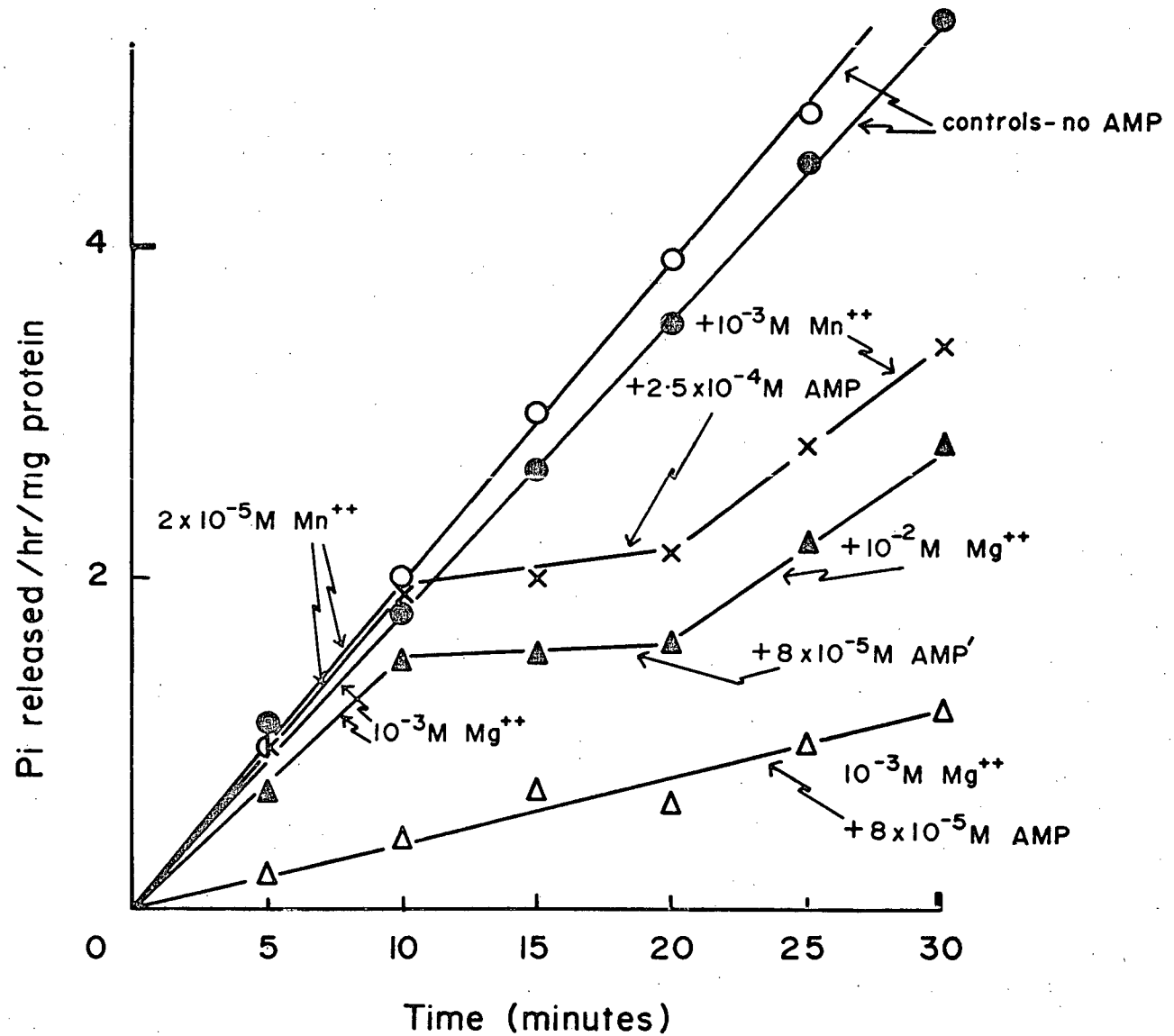
Concn. of cofactor (mM)		$10^4 K_i$ for AMP (M)	n	Probability of n being zero
Mg^{2+}	0.6	1.3	1.89	0.056
	1.0	2.1	1.49	0.081
	5.0	No inhibition		

site interaction, in this way regulating the effectiveness of AMP as an inhibitor. These results are obtained with either Mn^{2+} or Mg^{2+} in the reaction medium and provide a model for the control of FDPase activity.

Inhibition of the rat liver enzyme by AMP can be reversed by additional Mg^{2+} (Underwood & Newsholme, 1965a), and it appears that both Mn^{2+} and Mg^{2+} can partially, if not wholly, reverse AMP inhibition of the trout liver enzyme (Fig. 6). Thus 10 mM-Mg^{2+} restores about 70% of the former activity of FDPase that has been 80% inhibited by the addition of 80 µM-AMP, and 1 mM-Mn^{2+} restores about 50% of the original activity of FDPase that has been 80% inhibited by 0.25 mM-AMP (Fig.3.6). In contrast with FDPase from most other organisms (Horecker et al. 1966), rainbow-trout liver FDPase is inhibited by AMP at alkaline pH (Fig. 3.5). This result is not surprising, since the pH of the body fluids of fishes does not appear to be as rigidly controlled as that of mammals (see Steen, 1963). Here again the concentration of cofactor determines the apparent K_i (Table 3.IV and the degree of homotropic interaction between AMP-binding sites. The values of n at 1 mM-Mg^{2+} and 0.6 mM-Mg^{2+} are 1.49 and 1.89 respectively, which are in agreement with the calculated number of AMP-binding sites at pH 7.4.

Search for a metabolite activator of trout liver FDPase: A search was made for metabolic intermediates that would activate rainbow-trout liver FDPase. Considerable interest

Figure 3.6 Reversal of AMP inhibition of rainbow trout FDPase with both Mg^{2+} and Mn^{2+} as cofactors. Assays were carried out at pH 7.40, 15°C, and in the presence of 10^{-3} M FDP.


controls- no AMP
+10⁻³M Mn⁺⁺
+2·5x10⁻⁴M AMP
2x10⁻⁵M Mn⁺⁺
+10⁻²M Mg⁺⁺
+8x10⁻⁵M AMP'
10⁻³M Mg⁺⁺
10⁻³M Mg⁺⁺
+8x10⁻⁵M AMP
Pi released/hr/mg protein
Time (minutes)
0
5
10
15
20
25
30
2
4

centered on phosphoenolpyruvate and ATP, since the substrate of FDPase, FDP, is a positive modulator of rainbow-trout pyruvate kinase (Somero & Hochachka, 1968), and ATP is a negative modulator for fish phosphofructokinase (J. Freed, personal communication) and mammalian phosphofructokinase (Newsholme & Gevers, 1967).

The ability of phosphoenolpyruvate to act as a positive modulator towards FDPase would have made possible the realization of a coupling of two control sites in glycolysis and gluconeogenesis. Likewise, ATP activation of FDPase would couple with ATP inhibition of phosphofructokinase. However, the enzyme was not affected by either phosphoenolpyruvate or ATP, or by any other of the following metabolites tested: glucose 6-phosphate, 3-phosphoglycerate, pyruvate, lactate, oxaloacetate, malate, citrate, succinate, ADP, NADH. We therefore examined possible cationic control of the functions of rainbow-trout FDPase.

Cation requirements and cation control: Trout liver FDPase, like other types of FDPase examined (Gomori, 1943), shows an absolute requirement for a bivalent cation, which can be fulfilled by Mg^{2+} or Mn^{2+}. The Mg^{2+}-saturation curve is sigmoidal in the presence or absence of EDTA in the reaction medium (Fig.3.7). At high FDP concentration (1 mM) at 15°, K_a for Mg^{2+} of the rainbow-trout FDPase is 5×10^{-4} M. At 0.3 mM-FDP ($V_{opt.}$) the slopes of Hill plots for the rainbow-trout enzyme approximate to 2 at all temperatures except 0°, where n equals 0.924 (Table 3.V). These kinetics

Figure 3.7 Mg^{2+} saturation curves of rainbow trout FDPase at a series of temperatures. Assay conditions were as described in Fig. 4 except that the concentration of FDP was 3×10^{-4} M. Activity is expressed as 10^{-5} M P_i released/l./hr./mg. protein.

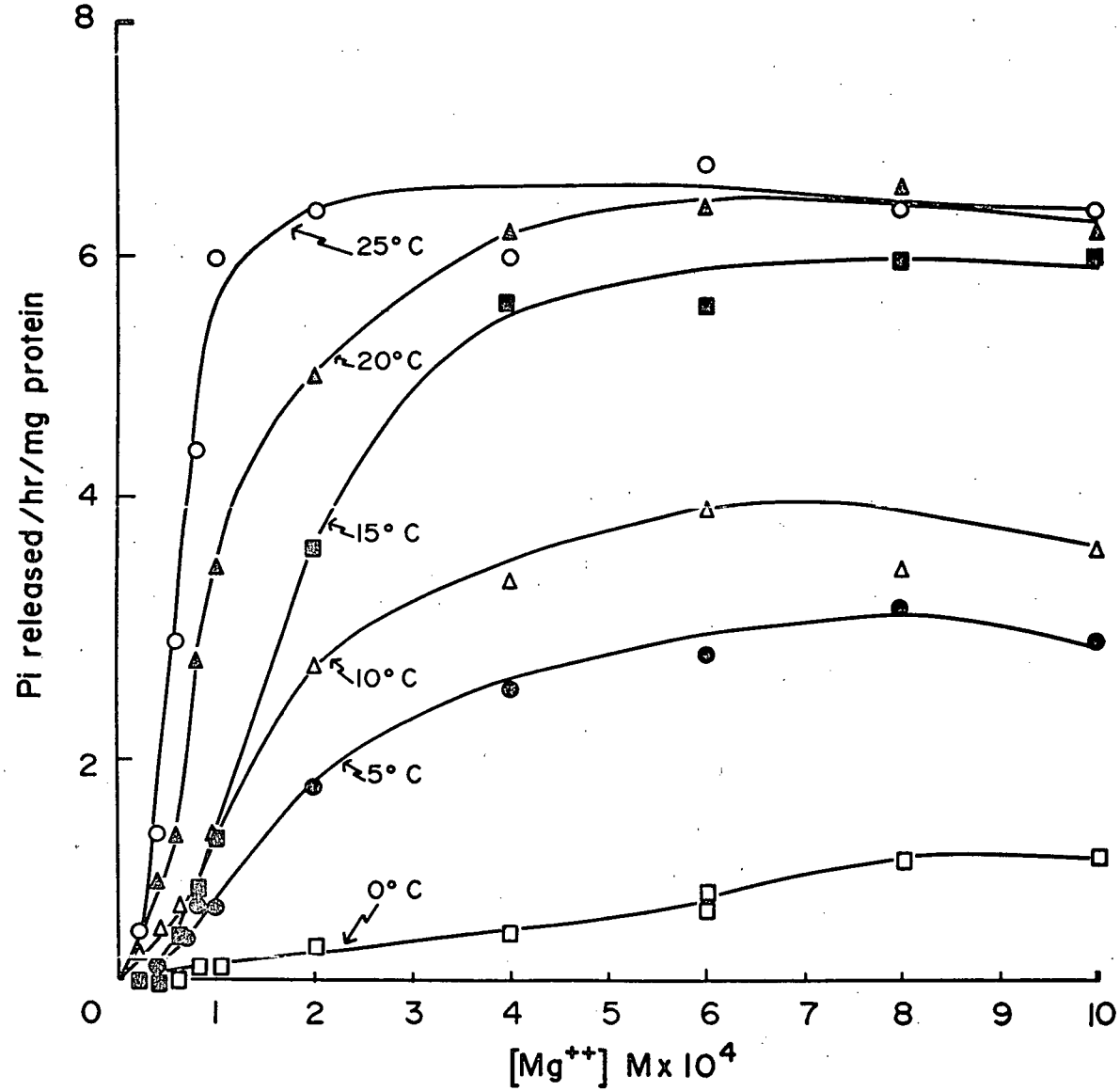

Pi released/hr/mg protein
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
[Mg++] M x 10^4
25° C
20° C
15° C
10° C
5° C
0° C

Table 3.V Effect of temperature on values of K_a and n of Mg^{2+}.

Assays were carried out in 0.05 M-tris-HCl, pH 7.40.

Temp.	0.3 mM-FDP 10^4K_a for Mg^{2+} (M)	n	Probability of n being zero	10^6K_a for Mn^{2+} (M)	1mM-FDP 10^6K_a for Mn^{2+} (M)
0°	5.2	0.924	0.006	3.5	
5	1.2	1.90	0.000		7.5
10	1.5	1.82	0.000	3.0	3.5
15	2.7	2.42	0.000	3.0	4.5
20	0.95	1.91	0.000	3.5	4.0
25	0.60	1.86	0.005	3.0	

suggest that FDPase contains interacting binding sites for Mg^{2+} and that Mg^{2+} serves both as a cation cofactor and as a positive modulator of the enzyme. The K_a for Mg^{2+} changes conspicuously with temperature (Table 3.V); there is a gradual fall in the value of K_a as the temperature is lowered from 15° to 5°, and then a sharp increase in K_a at 0°. The value of n for Mg^{2+} falls from 2 between 20° and 5° to less than 1 at 0°, indicating considerable decrease in the co-operative interaction between the Mg^{2+}-binding sites, a finding that may, in part, explain the large increase in K_a for Mg^{2+} at 0°. Then, as temperature rises from 15° to 25°, there is a rapid decrease in K_a for Mg^{2+}.

Under identical conditions (0.3 mM-FDP) the relationship between temperature and K_i for AMP is almost exactly opposite to the relationship between temperature and the K_a for Mg^{2+} (Tables 3.III & 3.V). The concentration of FDP also appears to affect the K_a for Mg^{2+}; thus, at 1 mM-FDP and 15^c, the K_a for Mg^{2+} is 4.5×10^{-4} M, whereas, at 0.3 mM-FDP and 15°, K_a for Mg^{2+} is 2.7×10^{-4} M.

The cation requirements of the enzyme can be satisfied by very much lower concentrations of Mn^{2+}. For rainbow-trout FDPase the Mn^{2+}-saturation curve is strictly hyperbolic and the K_a for Mn^{2+} (Fig.3.8) is about 50- to 100-fold lower than the K_a for Mg^{2+} (Fig.3.7) at all temperatures. The difference between Mg^{2+}- and Mn^{2+}-saturation curves is not understood. The results are similar to the effect of

Figure 3.8 Mn^{2+} saturation curves of rainbow trout FDPase at a series of temperatures. Assay conditions were as described in Fig. 4, except temperature was varied as indicated. Activity is expressed as 10^{-5} M P_i released/ l./hr./mg. protein.

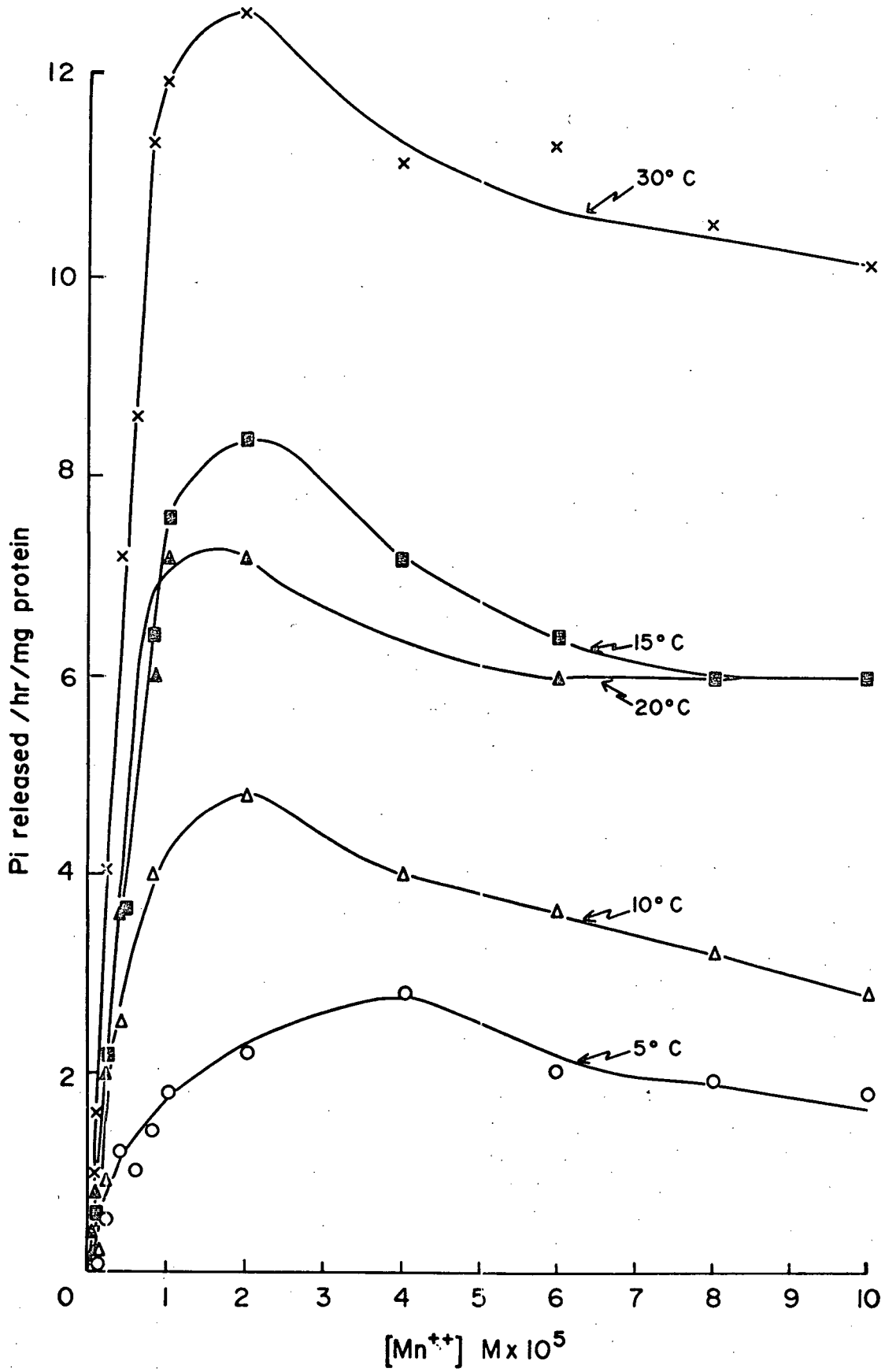

12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
Pi released /hr/mg protein
[Mn++] M x 10^5
30° C
15° C
20° C
10° C
5° C

Table 3.VI Inhibition of FDPase by Ca^{2+} and Zn^{2+} at various concentrations of cofactor.

Assays were carried out at 15° in 0.05 M-tris-HCl, pH 7.40, with 1mM-FDP in the reaction mixture.

Concn. of cofactor	$10^5 K_i$ for Ca^{2+} (m)	Concn. of cofactor	$10^7 K_i$ for Zn^{2+} (M)
Mg^{2+} 0.6 mM	2.8	Mg^{2+} 0.6 mM	1.5
1 mM	4.6	1 mM	4.8
5 mM	10.2	5 mM	5.0
10 mM	40.0		
Mn^{2+} 5 µM	23	Mn^{2+} 20 µM	7.4
10 µM	38	0.1 mM	9.6
20 µM	65		

Mn^{2+} on the FDP-activation curve of ADP pyrophosphorylase from E. coli B (Gentner, 1968): FDP is a positive modulator of this enzyme, and in the presence of Mg^{2+} the activation curve is sigmoid; when Mn^{2+} is present in the reaction mixture, the curve becomes hyperbolic. The concentrations of FDP affect the affinity of the rainbow-trout enzyme for Mn^{2+}. At all temperatures between 0° and 25°, K_a for Mn^{2+} is 3.0×10^{-6}-3.5×10^{-6} M at 0.3 mM-FDP ($v_{opt.}$). At high concentrations (1 mM) of FDP, K_a for Mn^{2+} varies slightly; at 5° K_a for Mn^{2+} rises to 7.5×10^{-6}M from a value of about 4×10^{-6} M at all other temperatures (Table 3.V). The enzyme is inhibited by higher concentrations of Mn^{2+} (Fig. 3.8); 0.1 mM-Mn^{2+} results in about 25% inhibition of activity at all temperatures.

Ca^{2+} is inhibitory to trout FDPase and is competitive with respect to both Mn^{2+} and Mg^{2+} (Fig.3.9). The K_i for Ca^{2+} depends on the Mn^{2+} or Mg^{2+} concentration (Table 3.II). At saturating, but not inhibitory, values of Mg^{2+}, the K_i for Ca^{2+} is about 4.5×10^{-5} M at 15°. Much higher concentrations of Ca^{2+} are required to compete with Mn^{2+}, presumably because of the high affinity of FDPase for Mn^{2+}; at saturating but not inhibitory concentrations of Mn^{2+} at 15°, the K_i for Ca^{2+} is about 8×10^{-4} M. Thus a given percentage inhibition in the presence of Mg^{2+} can be achieved by one-twentieth of the amount of Ca^{2+} that is required when Mn^{2+} is the cofactor.

Figure 3.9 Lineweaver-Burke plots of Ca^{2+} inhibition of rainbow trout FDPase showing the inhibition to be competitive with respect to the cofactor. Assay conditions were as described in Fig. 4.

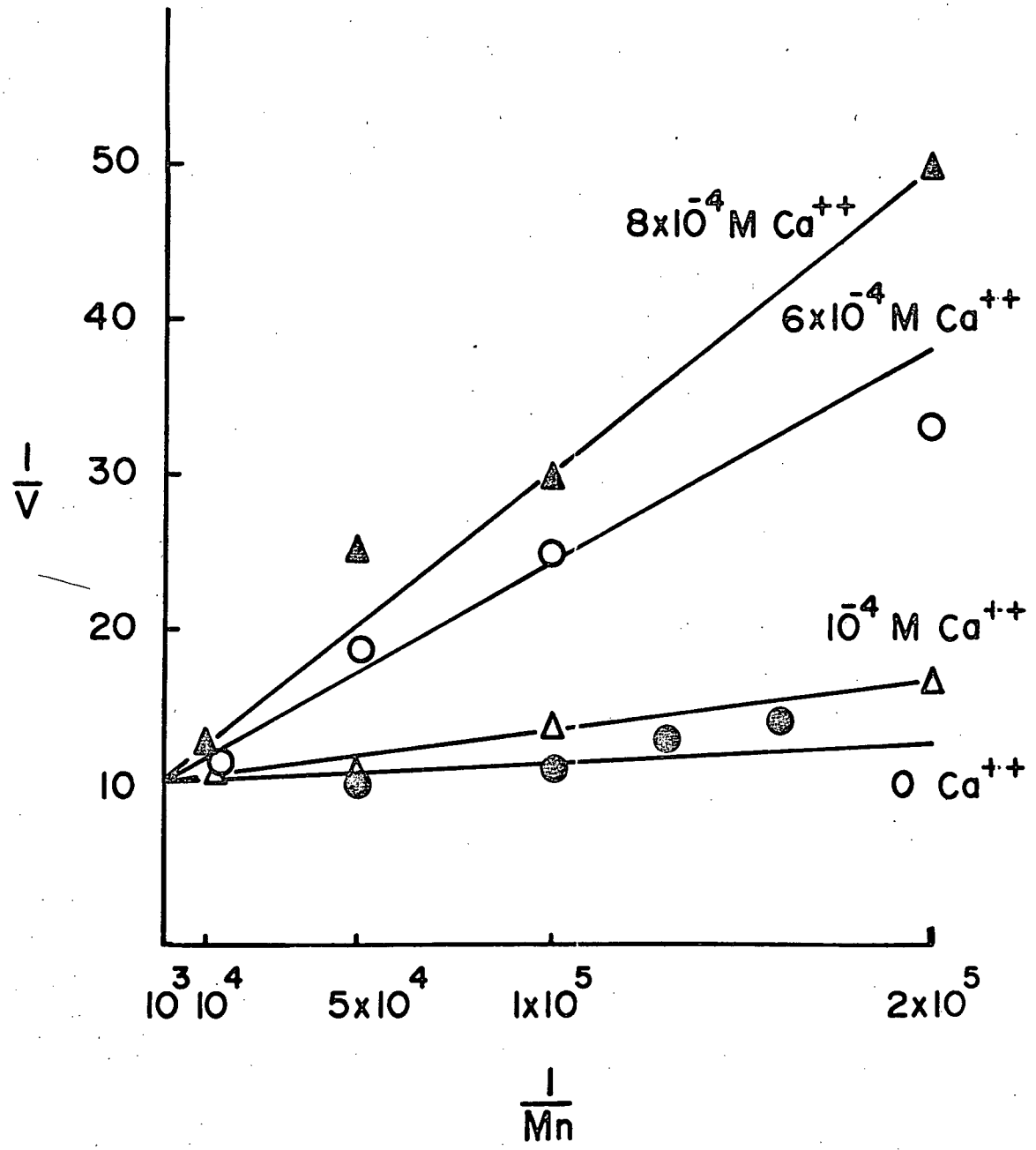
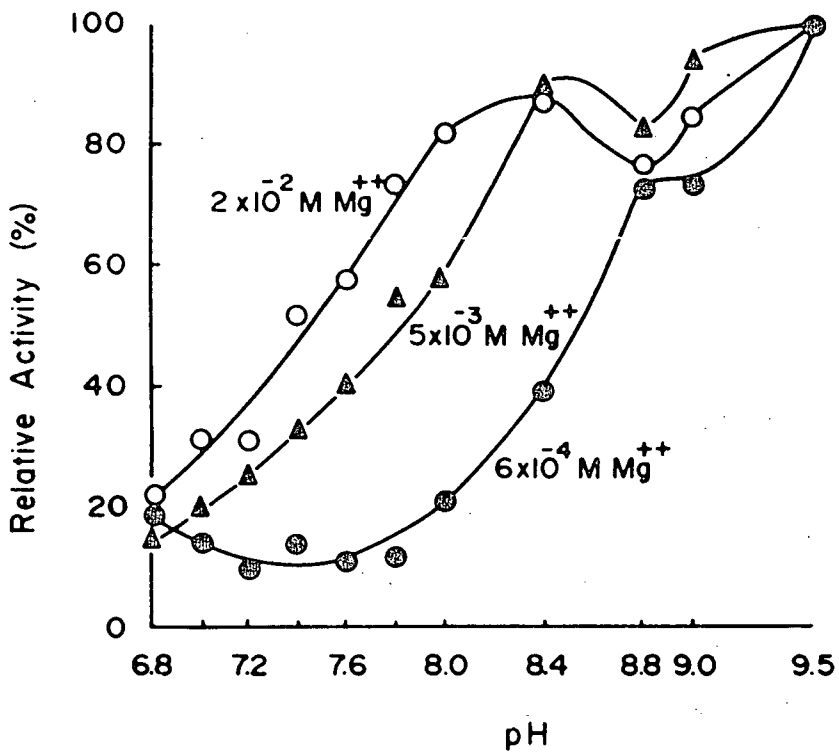
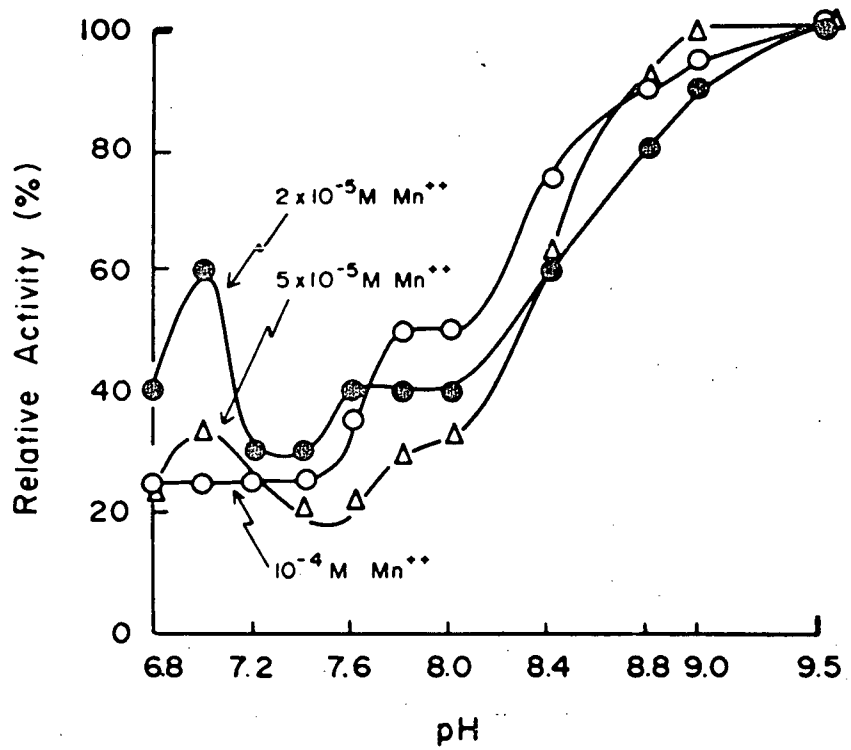

50
40
30
20
10
1/V
8x10⁻⁴ M Ca++
6x10⁻⁴ M Ca++
10⁻⁴ M Ca++
0 Ca++
10³ 10⁴
5x10⁴
1x10⁵
2x10⁵
1/Mn

Figure 3.10 Effects of changing concentrations of (a) Mg^{2+} and (b) Mn^{2+} on pH optima of rainbow trout FDPase. Assay conditions were as described in Fig. 4, except that pH was varied as indicated.

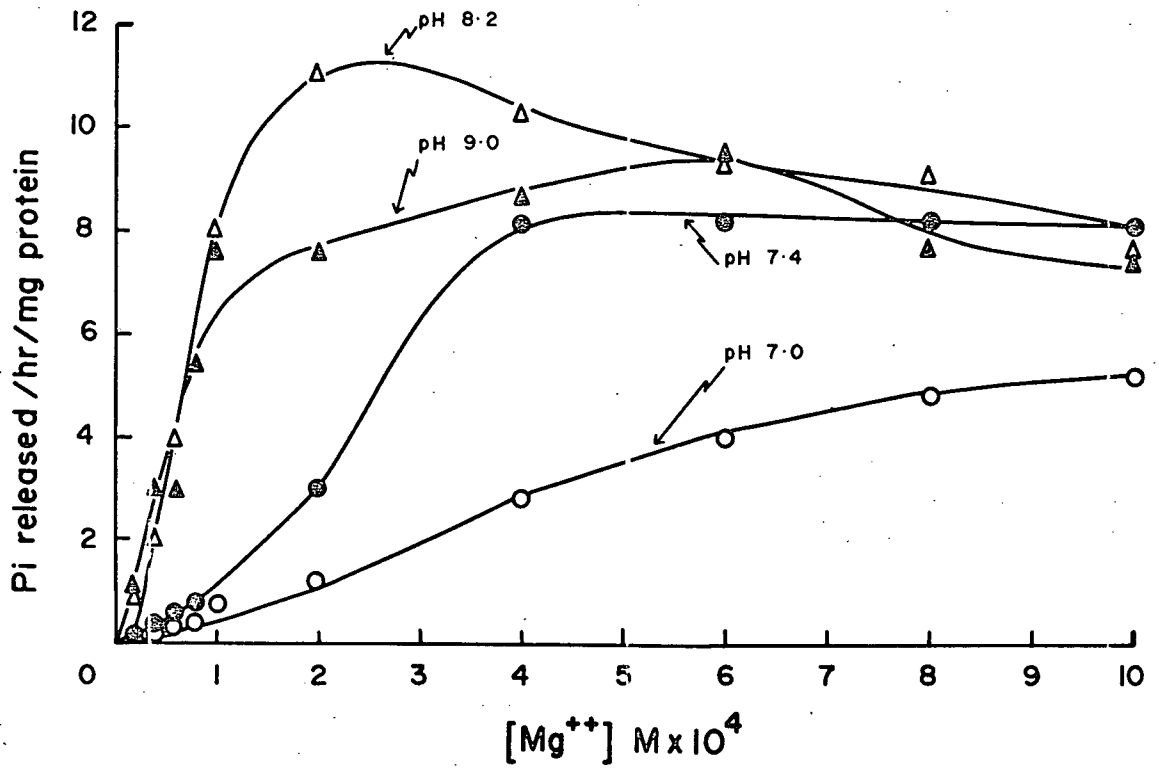

100
80
60
40
20
0
Relative Activity (%)
2 x10^{-2} M Mg^{++}
5x10^{-3} M Mg^{++}
6x10^{-4} M Mg^{++}
6.8
7.2
7.6
8.0
8.4
8.8
9.0
9.5
pH


100
80
60
40
20
0
Relative Activity (%)
2 x10^{-5}M Mn^{++}
5 x10^{-5}M Mn^{++}
10^{-4} M Mn^{++}
6.8
7.2
7.6
8.0
8.4
8.8
9.0
9.5
pH

The affinity of FDPase for Zn^{2+} is also very high. In the presence of saturating concentrations of Mn^{2+}, the K_i for Zn^{2+} is about 7×10^{-7} M, and in the presence of Mg^{2+}, the K_i for Zn^{2+} is about 5×10^{-7} M (see Table 3.VI) values that are well below the concentrations reported for tissue fluids (Krebs & Woodford, 1965).

As with most types of FDPase (Horecker et al. 1966; Byrne, 1964; Pontremoli, Luppis, Wood, Traniello & Horecker, 1965a; Pontremoli et al. 1965b), the pH curves are complex and the pH optimum for the rainbow-trout FDPase is alkaline and well beyond the expected physiological range at all concentrations of Mg^{2+}. High concentrations of Mg^{2+} tend to shift the pH optimum towards the physiological range (pH 7.5-7.8 for fish) and definitely stimulate FDPase activity at these pH values (Fig.3.10). This effect of Mg^{2+} on pH optima is even more marked for spinach leaf FDPase (Preiss et al. 1967), in which high Mg^{2+} concentrations decrease the optimum to about pH 7.4. The K_a for Mg^{2+} is also affected by pH (Fig.3.11); thus increasing pH tends to lower the K_a for Mg^{2+}. Hill plots of these data indicate n values for Mg^{2+} of about 2 at pH 7.0, pH 7.4 and pH 9.0. However, at pH 8.2, which appears to be an optimum for some of our preparations of the trout enzyme, the slope of the Hill plot is 3.04, indicating increased co-operation between the Mg^{2+}-binding sites (Table 3.VII). In the presence of Mn^{2+}, there is a distinct pH optimum at pH 9.0-9.5 at all Mn^{2+} concentrations (Fig.3.10). At lower concentrations of Mn^{2+}, another

Figure 3.11 Effect of pH on Ka's of Mg^{2+} and Mn^{2+} for rainbow trout FDPase. Assay conditions were as described in Fig. 4. FDPase activity is expressed as 10^{-5} M P_i released/l./hr./mg. protein.


pH 8·2
pH 9·0
pH 7·4
pH 7·0
Pi released /hr/mg protein
[Mg++] M x 10^4
0
1
2
3
4
5
6
7
8
9
10
2
4
6
8
10
12

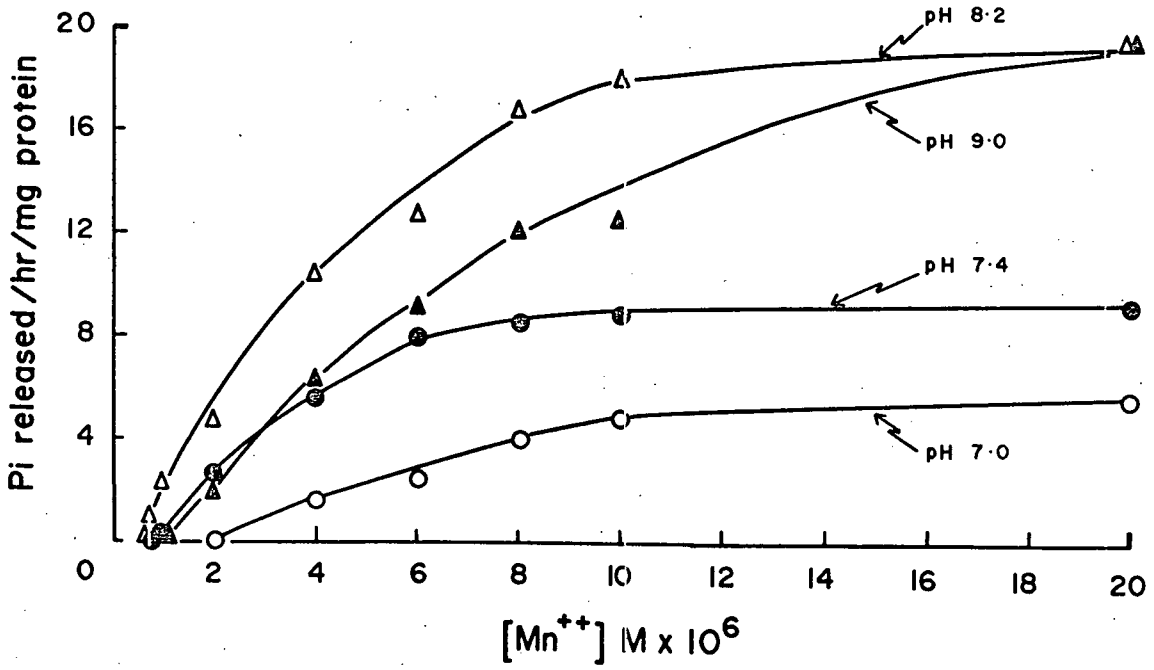

pH 8·2
pH 9·0
pH 7·4
pH 7·0
Pi released /hr/mg protein
[Mn++] M x 10^6
0
2
4
6
8
10
12
14
16
18
20
4
8
12
16
20

Table 3.VII Effect of pH on affinity of rainbow-trout FDPase for cofactor.

(Assays were carried out at 15° in the presence of 1 mM-FDP. Hill plots for Mn^{2+} saturation curves were not made because the Mn^{2+} curve is hyperbolic (see Fig. 8).

pH	$10^4 K_a$ for Mg^{2+} (M)	n	Probability of n being zero	$10^6 K_a$ for Mn^{2+} (M)
7.0	3.7	1.81	0.000	6.0
7.4	2.3	2.88	0.008	3.2
8.2	0.7	3.04	0.014	3.6
9.0	0.7	1.47	0.001	6.4

optimum is seen at pH 7.0, which disappears at inhibitory concentrations of the cofactor (Fig. 3.10).

DISCUSSION

These results clearly show that modulation of the concentrations of FDP, AMP, Mg^{2+}, Mn^{2+} and Ca^{2+} in the liver cell can markedly affect the activity of rainbow-trout liver FDPase and thus ultimately the production of glucose. The inhibition of FDPase and concomitant activation of Phosphofructokinase by AMP (Underwood & Newsholme, 1965a, b; Horecker et al. 1966) is important in the regulation of gluconeogenesis in pigeon liver homogenates (Gevers & Krebs, 1966). However, the remarkable temperature-dependence of FDPase-AMP interactions further complicates the problem in a poikilotherm. If AMP modulation were the major controlling mechanism, efficiency of metabolic control would be extremely sensitive to environmental temperature, and glucose production would be strongly inhibited at low temperatures. On the contrary, gluconeogenesis appears to be favoured during low-temperature acclimatization in fish (Hochachka, 1967). These conditions create a requirement for the control of sensitivity to AMP that can be satisfied by the ability of Mg^{2+} and Mn^{2+} to protect against AMP inhibition, to regulate AMP site-site interactions and to alter the pH optimum of the enzyme. These findings are of particular relevance to metabolic control in poikilotherms because Ca^{2+}, Mg^{2+} and probably Mn^{2+} are regulated during thermal acclimatization in fish (Rao, 1962; Hickman, McNabb, Nelson,

Van Breemen & Comfort, 1964; Houston & Madden, 1968. It is known that localized changes in cation concentration and compartmetation do occur in the cell (see Bygrave, 1967); hence changes in the kinetic characteristics of FDPase caused by such variations in cation concentrations may well be a major method of control of FDPase activity and gluconeogenesis.

CHAPTER 4

TEMPERATURE AND THE REGULATION OF ENZYME ACTIVITY IN POIKILOTHERMS: PROPERTIES OF LUNGFISH FRUCTOSE DIPHOSPHATASE

INTRODUCTION

Both the direction and rate of glucose carbon flow in glycolysis and gluconeogenesis in various animal tissues can be affected by the activity of FDPase (Weber, Singhal & Srivastava, 1965; Newsholme & Gevers, 1967). In all other systems examined (Newsholme & Gevers, 1967), with the exception of chloroplast FDPase (Morris, 1968), negative AMP modulation of FDPase is generally considered to be the major means of controlling the activity of this reaction. Thus conditions of energy saturation favour the FDPase reaction; energy-depleted conditions (when AMP concentrations are high) tend to inhibit the reaction. We became interested in examining the kinetic and regulatory properties of lungfish liver FDPase since lungfish maintain their liver glycogen contents during starvation and aestivation (Janssens, 1964), presumably through enhancement of gluconeogenesis from amino acid precursors (Janssens, 1964, 1965). The situation is further complicated by the fact that during

prolonged starvation (as in aestivation) the energy charge (Atkinson & Walton, 1967) of the cell may be decreased. This would lead to an inhibition of FDPase and would be inconsistent with maintenance of normal or even enhanced rates of gluconeogenesis that are observed during starvation (Weber *et al*. 1965). This suggests that other important control mechanisms are perhaps operative.

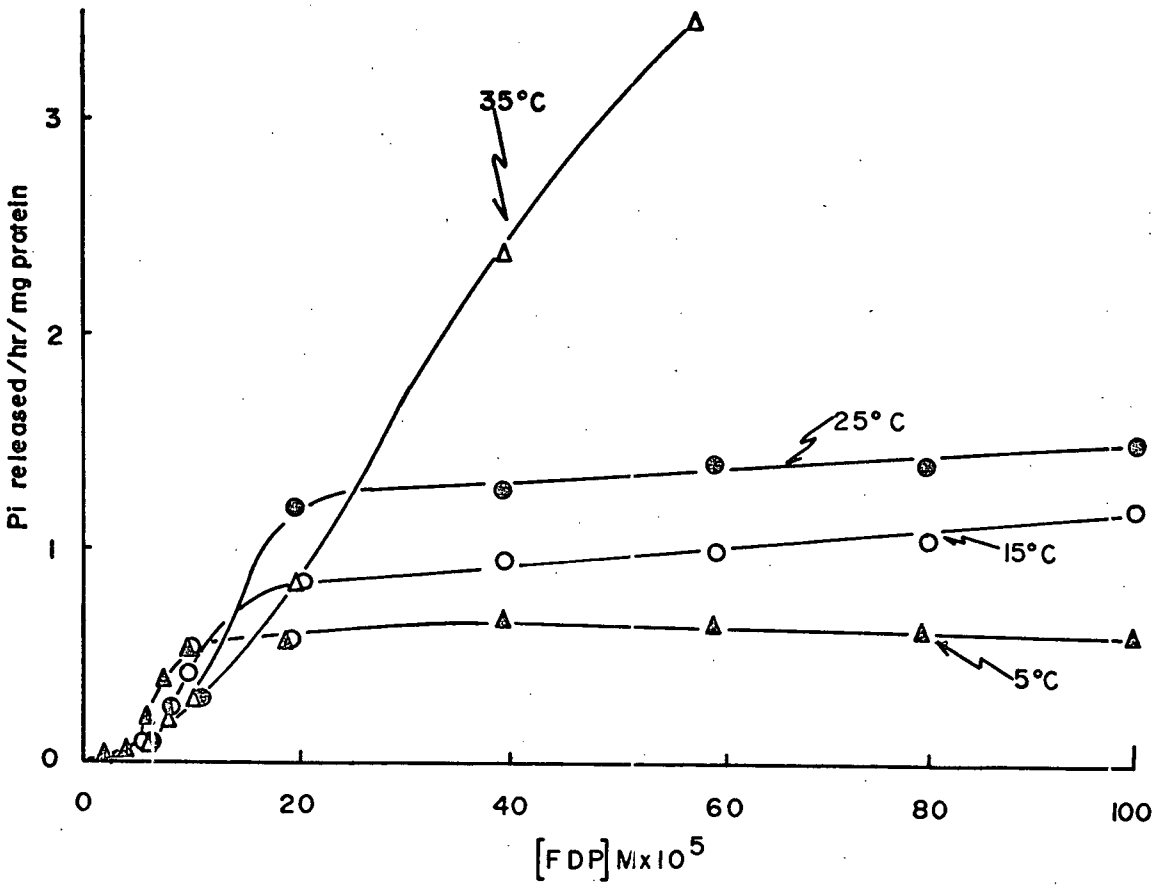
In addition, stimulation by pyruvate of liver glycogenesis in the South American lungfish appears to be favoured at temperatures in the upper thermal range of the animal (Hochachka, 1968). Since the kinetic and regulatory properties of FDPases from other organisms are highly temperature-sensitive (Taketa & Pogell, 1965; Behrisch & Hochachka, 1969), it was also decided to investigate the thermal properties of the lungfish enzyme.

RESULTS

The lungfish liver FDPase had a low affinity for its substrate (FDP) with either Mg^{2+} or Mn^{2+} in the reaction medium. The FDP saturation curves in the presence of either cofactor are sigmoidal and the Hill coefficients, n, which were calculated with a Fortran IV computer programme, are about 2 (Table 4.I). Since n is not an elementary kinetic parameter, but is a complex function of both the number of binding sites and the forces of interaction, the number of interacting sites that bind FDP is about two/molecule and cannot be given with any greater precision. In contrast with the FDPases from other organisms investigated (Horecker, Pontremoli, Rosen & Rosen, 1966; Underwood & Newsholme, 1965; Behrisch & Hochachka, 1969; Krebs, 1963), substrate inhibition is not seen in the lungfish enzyme (Fig. 4.1) in the presence of Mn^{2+}, and only about a 10 - 20% inhibition occurs at 1 mM-FDP in the presence of 1 mM-Mg^{2+} at 25° and 35° (Fig. 4.1).

Temperature does not appear to affect the affinity of lungfish FDPase for FDP with Mg^{2+} as cofactor between 5° and 25°; however, at 35° there is about a twofold increase in K_m for FDP (Table 4.I). Between 5° and 25°, at saturating FDP concentrations, values of Q_{10} are about 2; between 25° and 35° the Q_{10} increases to 2.6 (Table 4.II). When Mn^{2+} is present in the reaction medium, the affinity for FDP appears to display a more complex dependency on temperature

Figure 4.1 Substrate saturation curves of lungfish liver FDPase at different temperatures at pH 7.5. Activity is expressed as 10^{-5} M P_i released/ l./hr./mg. protein. The upper set of saturation curves were carried out in the presence of 8 x 10^{-5} M Mn^{2+} and the lower set of saturation curves in the presence of 1 mM Mg^{2+}.


Pi released/hr/mg protein
3
2
1
0
35°C
25° C
15°C
5°C
0
20
40
60
80
100
[FDP] M x 10^5

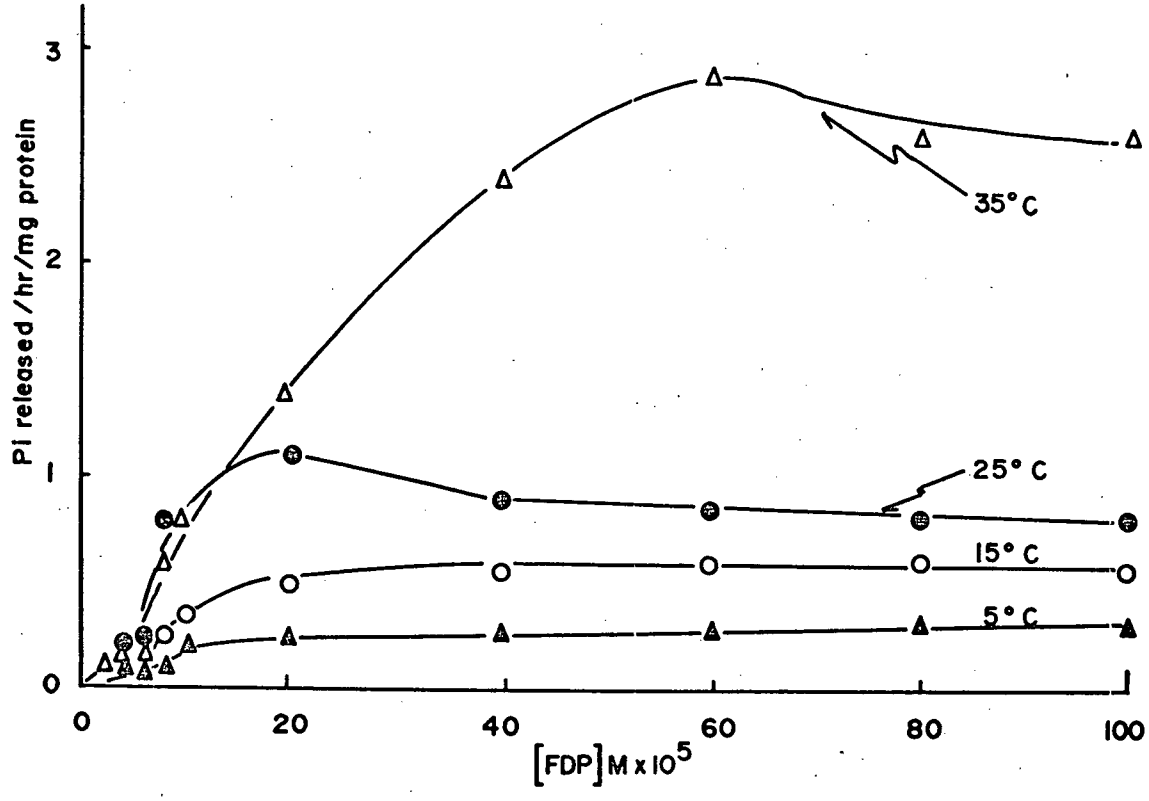

Pi released/hr/mg protein
3
2
1
0
35° C
25° C
15° C
5° C
0
20
40
60
80
100
[FDP] M x 10^5

Table 4.I Effect of temperature on affinity of lungfish FDPase for FDP.

Assays were carried out at pH 7.5. Values of n_H WERE calculated by a Fortran IV computer programme.

	1mM-Mg^{2+}			0.08mM-Mn^{2+}		
Temp.	10^5 K_m for FDP (M)	n_H	Probability of n_H being zero	10^5 K_m for FDP (M)	n_H	Probability of n_H being zero
5°	9	1.4	0.00	7	2.6	0.04
15	9	1.6	0.00	12	1.7	0.00
25	9	2.2	0.03	15	2.1	0.00
35	20	1.7	0.00	58	1.9	0.00

Table 4.II Activation energies and Q_{10} values of FDPase action with either Mg^{2+} or Mn^{2+} as cofactor.

Assays were carried out at pH 7.5. Activation energies were calculated from $V_{opt.}$.

	1 mM-Mg^{2+}		0.08 mM-Mn^{2+}	
Temp. range	Ea (kcal./mole)	Q_{10}	Ea (kcal./mole)	Q_{10}
5 - 15°	11.0	2.0	9.1	1.8
15 - 25	10.5	1.8	3.8	1.2
25 - 35	17.6	2.6	29.0	4.9

(Fig.4.1). Thus the apparent K_m for FDP rises from 7 x 10^{-5} M at 5° to 58 x 10^{-5} M at 35°. This large increase in K_m for substrate in the presence of Mn^{2+} is accompanied by about a fivefold increase in V_{max}. and a three- to eight-fold increase in the apparent activation energy (Table 4.II). Between 15° and 25° and with Mn^{2+} as cofactor the Q_{10} is less than 2.

The identity of the cationic cofactor also determines the activation energy of the enzyme over a particular temperature range (see Fig.4.2). Thus when 1 mM-Mg^{2+} is present in the reaction medium the activation energy is approximately 11 kcal./mole of enzyme between 15° and 25°, and about double this between 25° and 35°. On the other hand when optimum concentrations of Mn^{2+} are present in the reaction medium the enzyme appears relatively insensitive to temperature between 15° and 25°, but appears to be markedly stimulated between 25° and 35°. This result indirectly supports the finding by Hochachka (1968) that temperatures in the upper thermal range of the lungfish favour glycogenesis.

<u>Inhibition by AMP</u>: An important effector of FDPase is AMP (Taketa & Pogell, 1963, 1965; Underwood & Newsholme, 1965a; Wallace & Newsholme, 1967). The modulation of activity of FDPase by AMP has been shown to be highly dependent on temperature in the liver of rat (Taketa & Pogell, 1965), ox (Geller, 1967) and fish (Hochachka, 1967; Behrisch & Hochachka, 1969). Within the thermal range of the lungfish

Figure 4.2 Arrhenius plots of lungfish FDPase in the presence of optimum amounts of Mg^{2+} and Mn^{2+} at pH 7.5. The points are calculated from values of $V_{opt.}$.

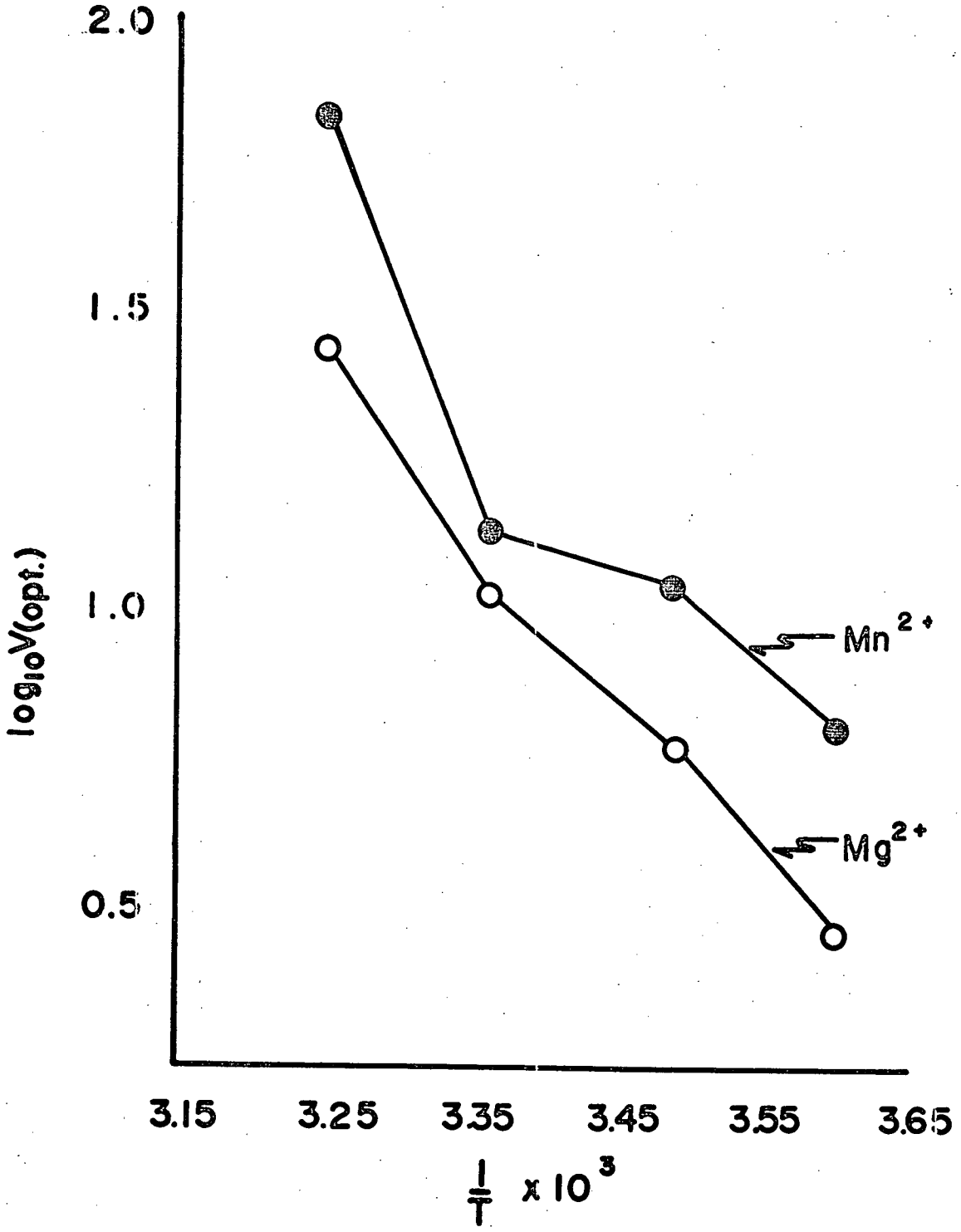
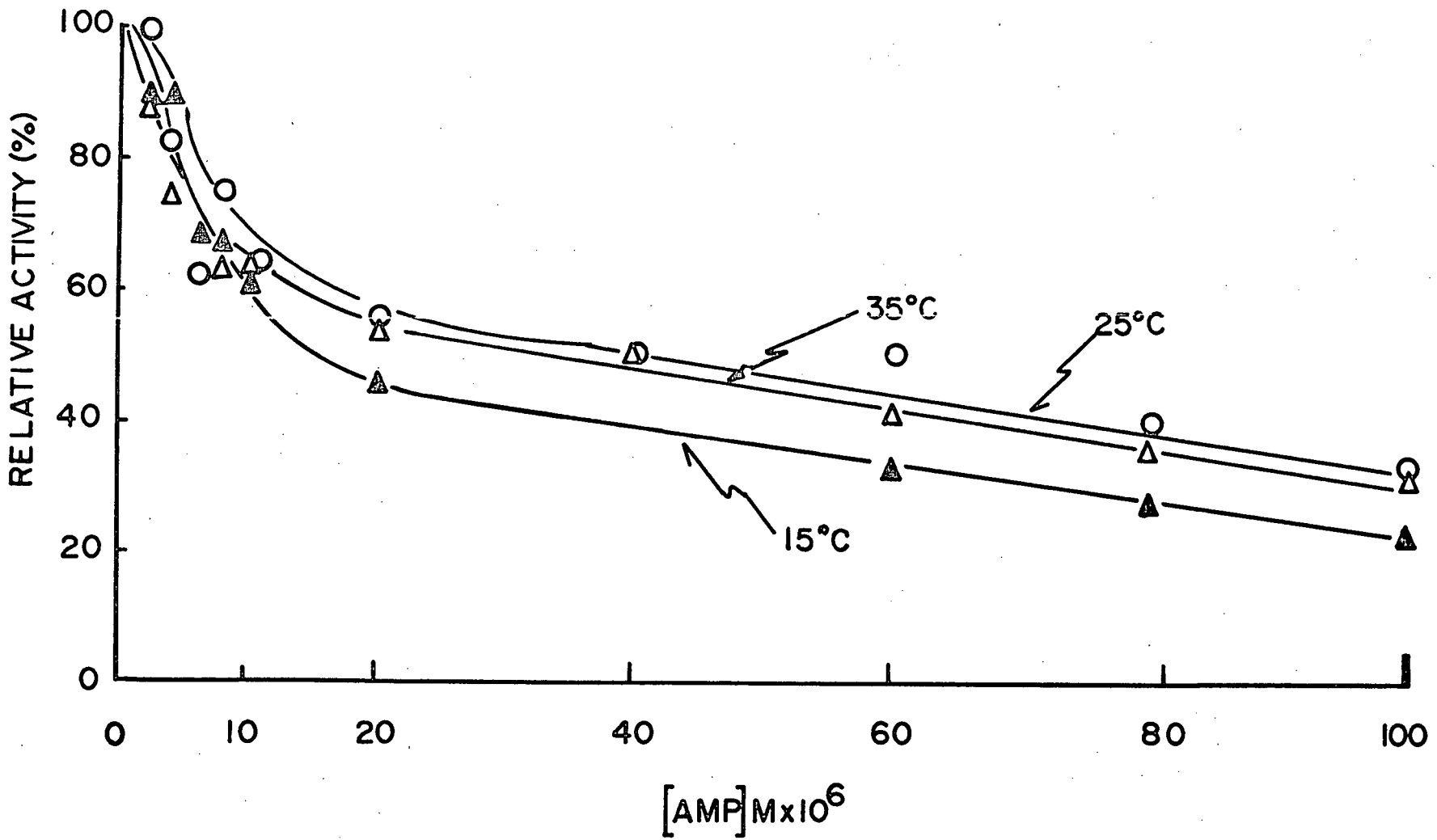

2.0
1.5
1.0
0.5
$\log_{10} V(\text{opt.})$
Mn^{2+}
Mg^{2+}
3.15
3.25
3.35
3.45
3.55
3.65
$\frac{1}{T} \times 10^3$

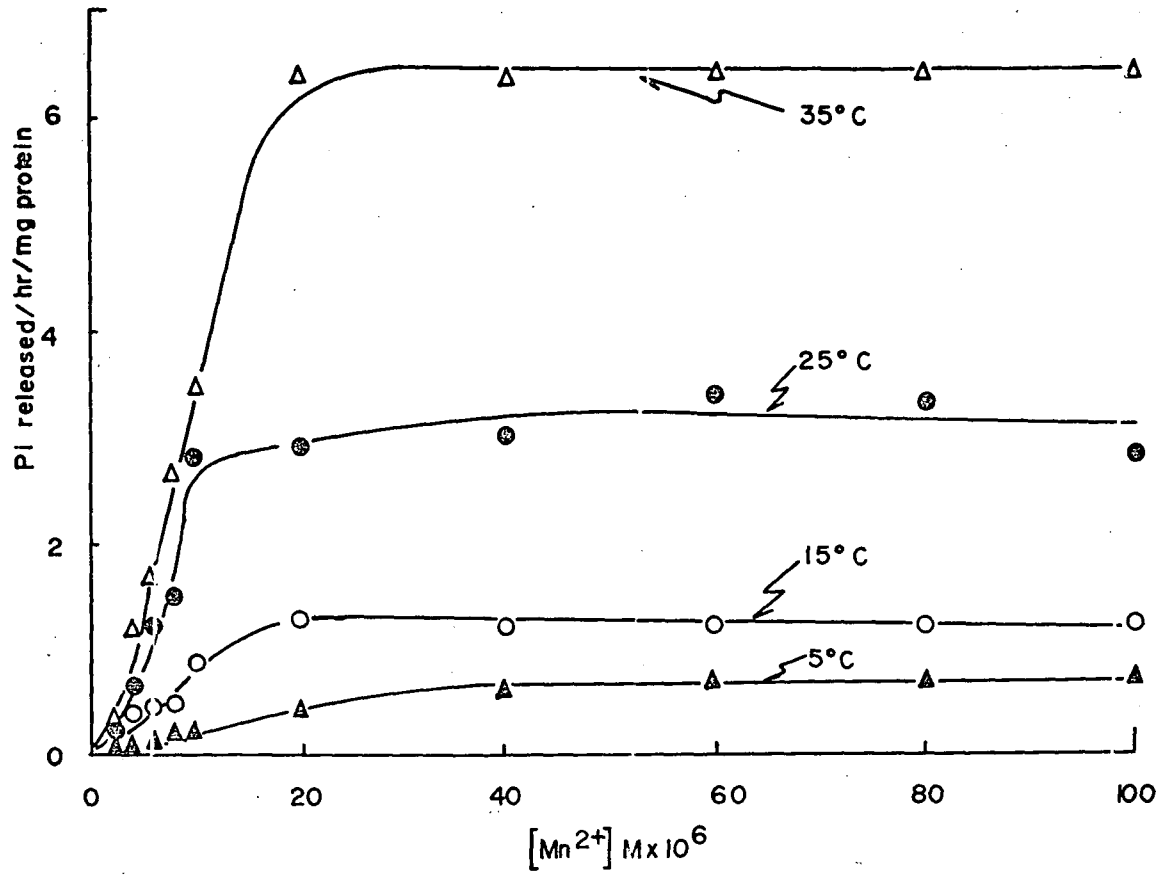
Figure 4.3 Inhibition of lungfish FDPase by AMP. Assay conditions were 1 mM FDP, 1mM Mg^{2+} and pH 7.5 and varying temperature. Activity is expressed as percent inhibition of total activity.


RELATIVE ACTIVITY (%)
100
80
60
40
20
0
0
10
20
40
60
80
100
[AMP] M x 10^6
35°C
25°C
15°C

and in the presence of optimum concentrations of Mg^{2+} the inhibition of FDPase by AMP appears to be almost totally independent of temperature (Fig.4.3), so that from between 15° to 40° the apparent K_i of AMP is about 3.5×10^{-5} M, some threefold higher than that for the trout FDPase (Behrisch & Hochachka, 1969). In addition, Hill coefficients of inhibition by AMP are about 1 at all temperatures (Table 4.III), indicating negligible co-operation between the AMP-binding sites. This finding provides further support for the hypothesis that AMP inhibition of FDPase activity in the lungfish is relatively insensitive to temperature. This is in complete contrast with inhibition by AMP of the trout enzyme in which the ability of AMP to act as an allosteric effector is highly dependent on temperature (Hochachka, 1967; Behrisch & Hochachka, 1969).

Cation requirements and cation control: Like all FDPases previously examined (Horecker *et al*. 1966) lungfish FDPase has an absolute requirement for a bivalent cation, which may be satisfied by either Mg^{2+} or Mn^{2+}. The saturation curve for Mg^{2+} is sigmoidal in the presence of optimum concentrations of FDP (Fig.4.4) at pH 7.5, and the K_a of Mg^{2+} does not appear to change appreciably with temperature. From the Hill coefficient (Table 4.IV) the apparent number of interacting sites that bind Mg^{2+} is about two/molecule of enzyme. The cation requirements of the enzyme may be fulfilled by concentrations of Mn^{2+} that are about 10 - 30 fold lower (Table 4.IV) than those of Mg^{2+}. The saturation

Figure 4.4 Mg^{2+} and Mn^{2+} saturation curves of lungfish FDPase at a series of temperatures. Assay conditions were 1 mM FDP and pH 7.5. Activity is expressed as 10^{-5} M P_i released/l./hr./mg. protein.


PI released/hr/mg protein
6
4
2
0
35° C
25° C
15° C
5°C
0
20
40
60
80
100
[Mn2+] M x 10^6

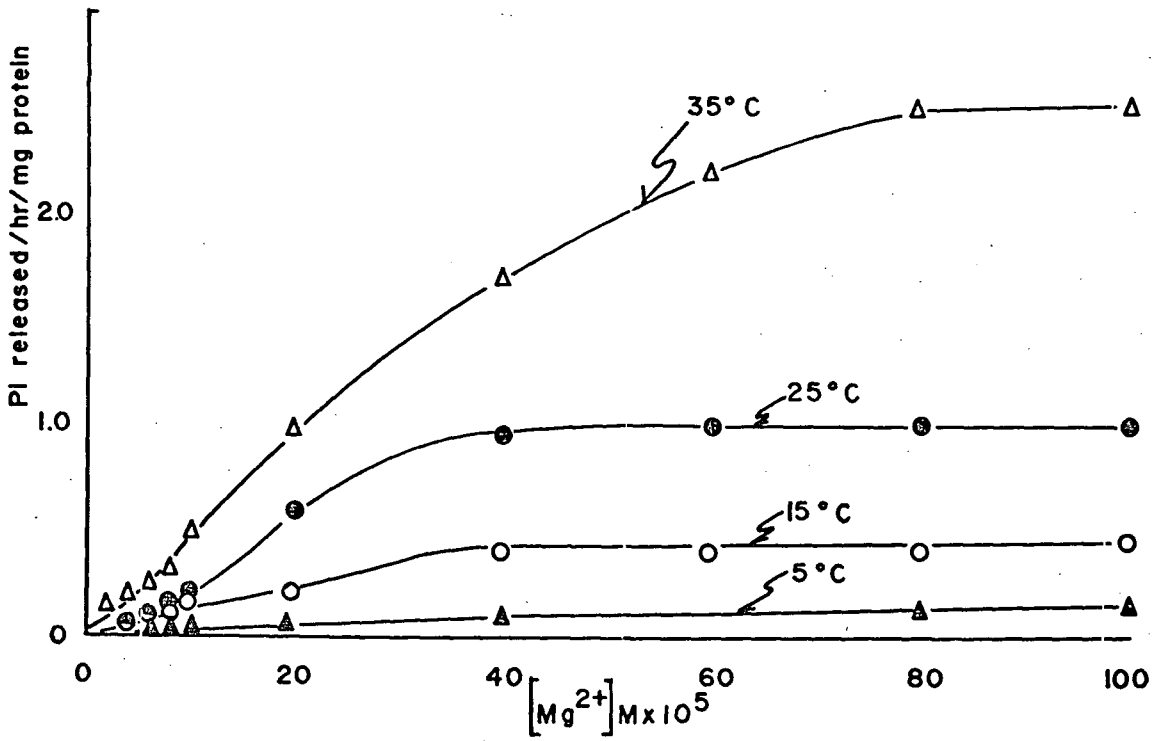

PI released/hr/mg protein
2.0
1.0
0
35° C
25° C
15° C
5° C
0
20
40
60
80
100
[Mg2+] M x 10^5

Table 4.III Effect of temperature on inhibition of lungfish FDPase by AMP.

Activities were measured in the presence of 1 mM-Mg^{2+} with 1 mM-FDP at pH 7.5.

Temp.	10^5 K_i for AMP (M)	n_H	Probability of n_H being zero
15	1.5	0.85	0.00
25	3.5	0.91	0.00
35	3.4	0.58	0.00
40	3.4	0.87	0.00

Table 4.IV Effect of temperature on affinity of lungfish FDPase for cation cofactor.

Assays were carried out with 1 mM-FDP at pH 7.5

Temp.	10^4 K_a for Mg^{2+} (M)	n_H	Probability of n_H being zero	10^6 K_a for Mn^{2+} (M)	n_H	Probability of nH being zero
5°	2.5	0.6	0.21	16	1.7	0.00
15	2.0	1.3	0.00	7	1.3	0.03
25	2.8	1.4	0.02	7	1.7	0.00
35	2.5	1.4	0.00	9	1.9	0.00

curve for Mn^{2+} is sigmoid (Fig.4.4), in contrast with the hyperbolic saturation curve for Mn^{2+} of the rainbow-trout FDPase (Behrisch & Hochachka, 1969). As with Mg^{2+} the values of the Hill coefficients for Mn^{2+} binding approximate to 2, and this value appears to be independent of temperature.

Lungfish FDPase has a distinct alkaline pH optimum in the presence of optimum concentrations of Mg^{2+} (Fig.4.5). However, at optimum concentrations of Mn^{2+} the pH-activity profile changes markedly (Fig.4.5). There is a broad peak of maximum or near-maximum activity extending from pH 7.5 to pH 9.5. Replacement of Mg^{2+} with Mn^{2+} appears to stimulate activity near the neutral end of the pH range, a finding not obtained with the rainbow-trout enzyme (Behrisch & Hochachka, 1969). The affinity of lungfish FDPase for its cofactors seems to be dependent on the pH (Table 4.5), with a rise in pH causing a decrease in the K_a values of Mg^{2+} and Mn^{2+} (Fig.4.6) and a marked increase in $V_{max.}$. At high pH (pH 9.0) the sigmoidicity of the saturation curves for Mn^{2+} and Mg^{2+} disappears and the saturation cruves become hyperbolic, a finding that is quite similar to the effect of pH on the saturation curves for Mg^{2+} of rainbow-trout FDPase (Behrisch & Hochachka, 1969).

Figure 4.5 Effect of pH on activity of lungfish FDPase with Mg^{2+} and Mn^{2+} in the reaction medium. Assay conditions were 1 mM FDP and 25°C. Activity is expressed as 10^{-5} M P_i released/ l./hr./mg. protein.

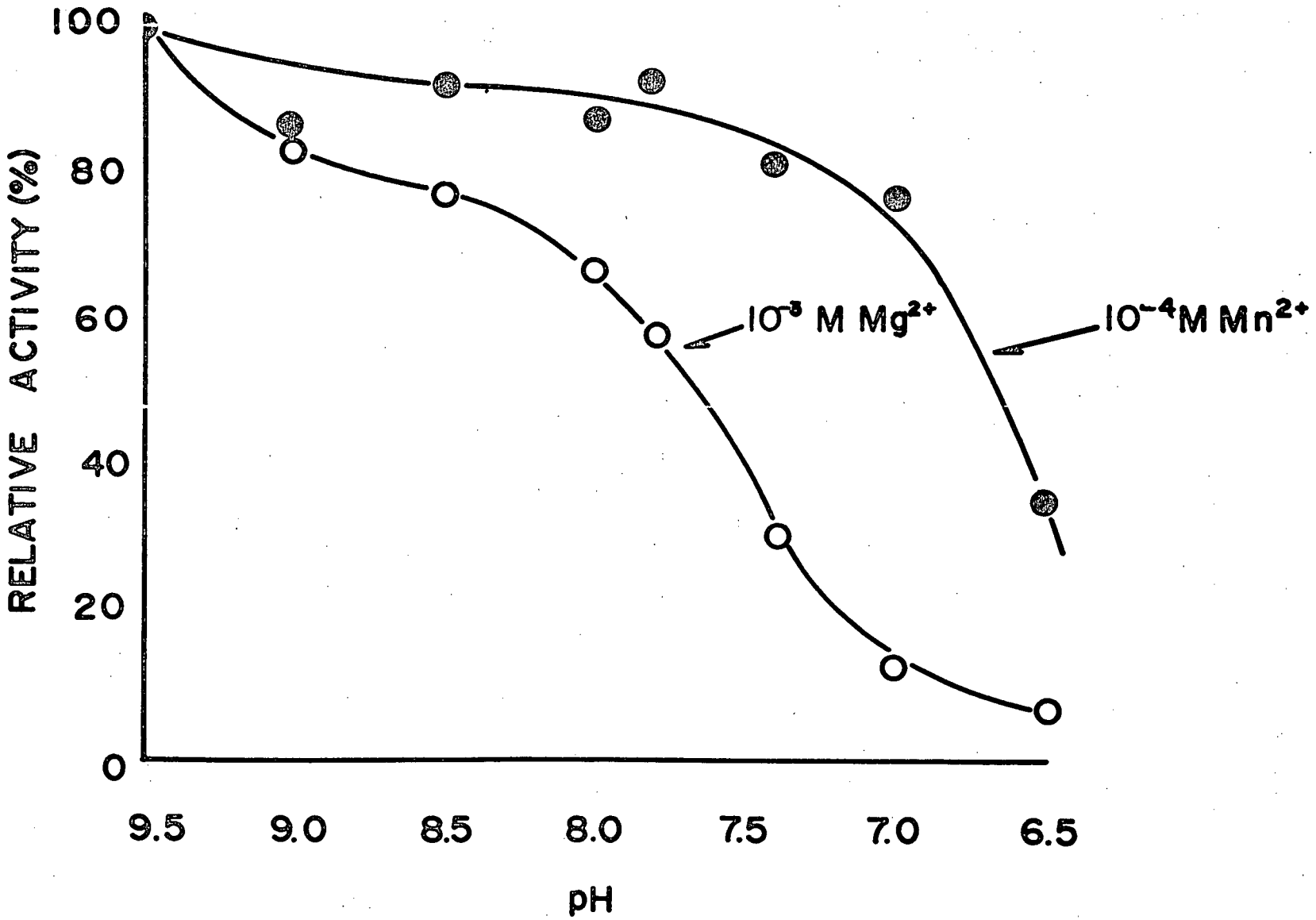

100
80
60
40
20
0
RELATIVE ACTIVITY (%)
10^{-3} M Mg^{2+}
10^{-4}M Mn^{2+}
9.5
9.0
8.5
8.0
7.5
7.0
6.5
pH

Table 4.V Effect of pH on affinity of FDPase for cation cofactor.

Assays were carried out with 1 mM-FDP at 27°.

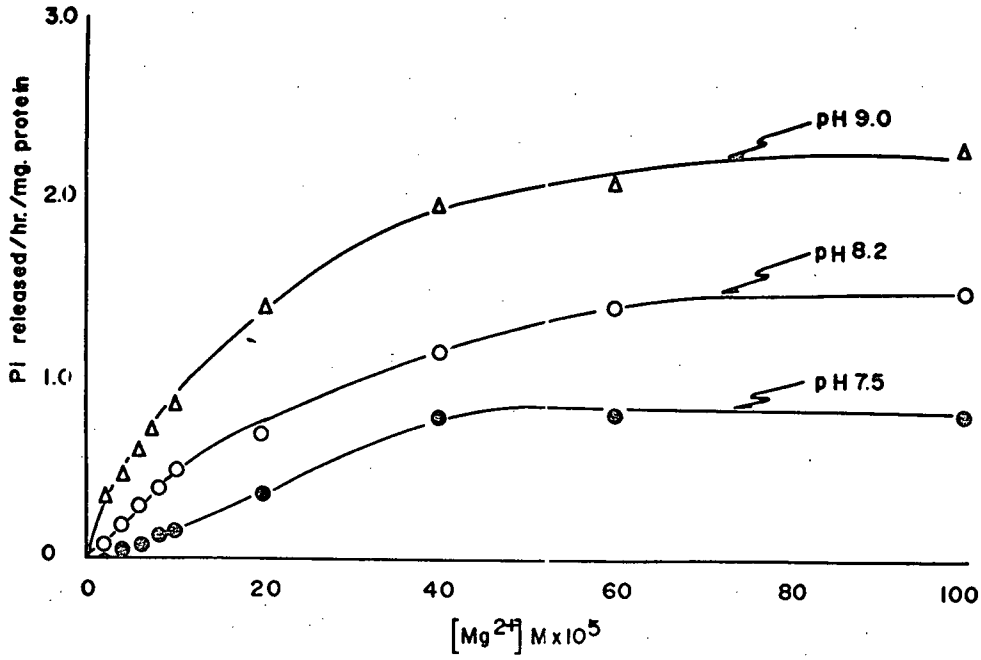
pH	10^4 K_a for Mg^{2+} (M)	n_H	Probability of n_H being zero	10^6 K_a for Mn^{2+} (M)	n_H	Probability of n_H being zero
7.5	2.2	2.3	0.00	24	1.9	0.00
8.2	2.0	1.5	0.00	11	1.7	0.00
9.0	1.6	1.3	0.00	8	1.6	0.00

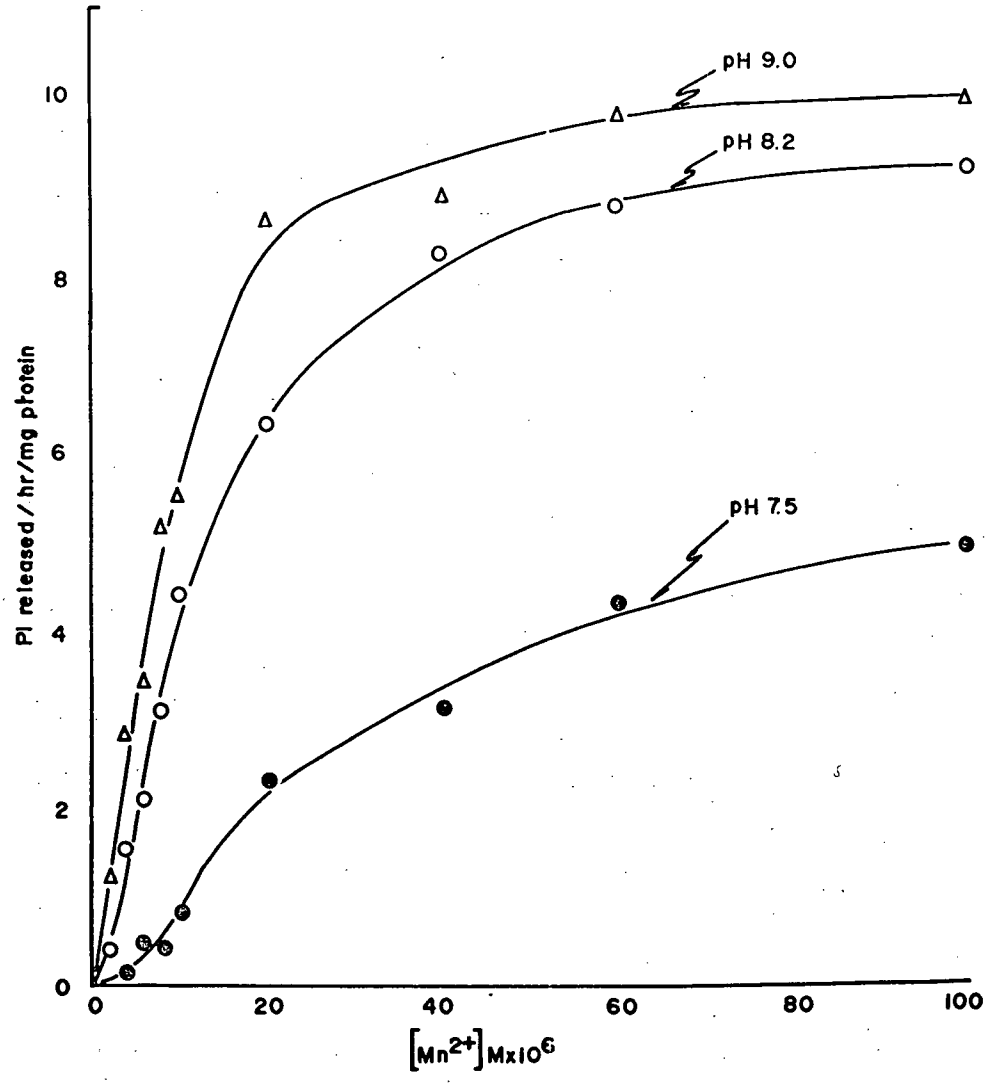
DISCUSSION

It is generally accepted that the major means of regulation of FDPase is by negative modulation by AMP (see Atkinson, 1966; Horecker et al. 1966). During prolonged starvation or aestivation in the lungfish energy charge of the liver cell may be expected to be depressed, and, since the K_i for AMP is quite low, this condition would tend to maintain FDPase in a "shut-off" conformation. However, in aestivation or starvation maintenance and regulation of liver glucose and glycogen contents would seem to be a condition of survival (Janssens, 1964) requiring normal or indeed relatively enhanced FDPase activity (Weber et al. 1965). Hence one might predict a requirement, under these conditions, for mechanisms of activation of FDPase or a means of releasing the system from control by AMP inhibition.

It is known from previous studies (Underwood & Newsholme, 1965a; Wallace & Newsholme, 1967) that Mg^{2+} can reverse AMP inhibition, but in fish systems the changes in concentration of Mg^{2+} required are quite high (Behrisch & Hochachka, 1969a) and this would not seem to be a plausible control feature. Another means of regulating FDPase involves H^+. As is evident in Fig. 4.), increasing pH can lead to very dramatic changes in the K_a for Mg^{2+} (or Mn^{2+}) as well as in $V_{max.}$. Moreover, the pH-activity profile displays a steep section in the neutral range in the presence of Mg^{2+} or Mn^{2+}. Hence small changes in pH in this range can lead

Figure 4.6 Effect of pH on affinity of lungfish FDPase for cofactor. Assay conditions were 1 mM FDP_6 and 27°C. Activity is expressed as 10^{-5} M P_i released/l./hr./mg. protein.


3.0
2.0
1.0
0
Pi released/hr./mg. protein
pH 9.0
pH 8.2
pH 7.5
0
20
40
60
80
100
[Mg2+] M x 10^5


10
8
6
4
2
0
Pi released / hr/mg protein
pH 9.0
pH 8.2
pH 7.5
0
20
40
60
80
100
[Mn2+] M x 10^6

to large changes in activity, a characteristic feature of many efficient regulatory metabolites (Atkinson, 1966). A very active phosphofructokinase could lead to local acidification, since H^+ is a product of the reaction. Conversely a low rate of glycolysis and phosphofructokinase activity, such as might be expected during lungfish aestivation, would favour a rise in pH and so lead to a significant activation of FDPase.

Although previous studies of FDPase have considered the pH-activity profile of the enzyme in some depth (Horecker et al. 1966), the importance of this type of control has been largely overlooked. Preiss, Biggs & Greenberg (1967) have shown that the pH requirements of the FDPase from spinach leaves can be strongly affected by changes in concentration of Mg^{2+} and likewise indicate a rather steep section in the pH-activity profile. Similar results have been obtained with trout liver FDPase (Behrisch & Hochachka, 1969a). In this connection, Trivedi & Danforth (1967) observed profound effects of pH on the phosphofructokinase reaction, which may couple, in the manner indicated above, with the effect of H^+ on activity of FDPase. Such a coupling of the control of phosphofructokinase and FDPase is analogous to the coupling of adenylate control of these two enzymes: conditions that favour activity of phosphofructokinase favour inhibition of FDPase, and conditions that inhibit phosphofructokinase tend to activate FDPase (Atkinson, 1966).

A third mechanism for activation of FDPase may involve the substrate, FDP. In previous studies FDP has been implicated in the control of interconversion of FDP and fructose 6-phosphate by the complementary mechanisms of substrate inhibition of FDPase and product activation of phosphofructokinase (see Atkinson, 1966). However, Newsholme & Gevers (1967) argue that substrate inhibition of FDPase is probably not of physiological significance because the concentrations of FDP required are high and the percentage inhibition is relatively low. Recent studies (Preiss et al. 1967; Behrisch & Hochachka, 1969a) indicate that at least under certain conditions the FDP saturation curve is sigmoidal, suggesting co-operative interaction between substrate-binding sites. Since the apparent K_m for FDP for the lungfish and trout enzymes appears to fall well within the physiological concentration ranges for the substrate (Williamson, Cheung, Coles & Herczeg, 1967), it appears that FDP can serve as a positive modulator as well as substrate of FDPase.

Often it has been suggested that in poikilotherms evolutionary adaptation to temperature may involve selection for enzymes of greater "efficiency" than in warm-blooded animals. In earlier studies the most commonly used measure of "efficiency" was the activation energy of the given enzyme-catalysed reaction, the implication being that enzymes with lower activation energies would be favoured in environments of low thermal energy. In our comparative

analyses we have been unable to find such a correlation consistently (Hochachka & Somero, 1968; Somero & Hochachka, 1968). Moreover, for a large number of enzymes, including FDPases from trout (Behrisch & Hochachka, 1969a), salmon (H.W. Behrisch, unpublished work) and the lungfish (Fig.4.2), Arrhenius plots are often complex and can be affected by substrates or modulators (Lowry, Schulz & Passonneau, 1964; Helmreich & Cori, 1964; Massey, Curti & Ganther, 1966). For these reasons (for further discussion of this matter in relation to poikilothermy see Hochachka & Somero, 1968) it appears that in evolutionary adaptation to unique thermal environments enzyme "efficiency" as measured by activation energy does not appear to be a fundamental site of natural selection. Rather, the sites most sensitive to selective pressure appear to include (1) enzyme affinity for unique substrate (Somero & Hochachka, 1968) and (2) enzyme affinity for cofactors or modulators or both (Atkinson & Walton, 1967; Behrisch & Hochachka, 1969a).

In a physiological sense the effect of temperature-dependent changes in enzyme-ligand affinities appears to be a decrease in the Q_{10} of the reaction, whereas control mechanisms are held normally effective at all temperatures encountered in Nature. Thus with trout pyruvate kinase (Somero & Hochachka, 1968) the effect of a fall in temperature on reaction rate can be compensated (at physiological substrate concentrations) by a concomitant increase in enzyme affinity for substrate; on the other hand

control of the reaction by feedforward activation and feedback inhibition is largely temperature-insensitive. To some extent lungfish FDPase resembles trout pyruvate kinase in its temperature characteristics. Thus a decrease in temperature leads to an increase in affinity for FDP, whereas the K_i for AMP is largely independent of temperature. In these organisms, then, evolutionary "strategy" appears to be directed towards a gain of independence from thermal vagaries of the outer environment.

An alternative evolutionary "strategy", to take advantage of certain temperature variations, is implicit in the work on pyruvate kinases and lactate dehydrogenases (Somero & Hochachka, 1968; Hochachka & Somero, 1968). At the lower temperature extremes the K_m values for substrates for both enzymes fall dramatically as temperature rises. At normal tissue concentrations of these substrates a small increase in thermal energy in this temperature range can lead to a large increase in the reaction rate. By this means a substantial enzyme activity is achieved at temperatures only a few degrees above the lower lethal limit for the organism. A similar situation is not found with lungfish liver FDPase. Possibly one would not expect it, for the lungfish, as an evolving species, has not been faced with serious falls in temperature in its environment for many geological ages, if ever.

CHAPTER 5

TEMPERATURE AND THE REGULATION OF ENZYME ACTIVITY IN POIKILOTHERMS: FRUCTOSE DIPHOSPHATASE FROM MIGRATING SALMON

INTRODUCTION

The resynthesis of glucose from other sources is essential for the maintenance of certain types of cells and as a supply for immediate energy demands of the organism. Control of the activity of fructose 1,6-diphosphatase (catalyzing the conversion of FDP $\rightleftharpoons$ F-6-P + P_i) is essential since the simultaneous operation of both FDPase and phosphofructokinase (catalyzing the conversion of F-6-P + ATP FDP) could lead to a net hydrolysis of ATP and thus to a short-circuit in metabolism (Horecker *et al*. 1966). In all animal systems thus far considered (Newsholme and Gevers, 1967), negative modulation by 5'-AMP of FDPase is considered to be the major means of controlling the activity of this reaction. Thus, conditions of energy saturation would favour the FDPase reaction, and energy depleted conditions (when AMP concentrations are high) would tend to inhibit the reaction. This is a situation that may be true of biosynthetic reactions in general (Atkinson, 1968).

Control of FDPase activity in migrating salmon is complicated by two factors. First, salmon which are migrating upstream to the spawning grounds do not feed, so that energy is supplied through metabolism of fats and amino acids (Idler et al. 1959; Chang and Idler, 1960).

The extended starvation is further complicated by a high degree of muscular and biosynthetic activity in these fish. Such an energy-expending situation possibly leads to a reduced energy charge (Atkinson and Walton, 1967), which in other organisms favours inhibition of FDPase and gluconeogenic activity. This inhibition must be circumvented in order to maintain normal concentrations of blood glucose.

Second, during the upstream migration a salmon can experience rather marked changes in environmental temperature, particularly during the summer run, when depth of stream can change abruptly and temperature fluctuations may be quite pronounced. The allosteric inhibition by AMP of FDPase has been shown to be markedly sensitive to temperature, so that a decrease in temperature can result in a dramatic increase in affinity of the enzyme for its negative modulator (Taketa and Pogell, 1965; Hochachka, 1967; Behrisch and Hochachka, 1969a). This would mean that control of FDPase activity by AMP would be unusually sensitive to changes in temperature at a time when, in fact, there is a requirement for an active FDPase.

The above conditions of extended starvation, with concomitant physical and biosynthetic work and a variable

environmental temperature imply that effective regulatory mechanisms are available for reversing AMP inhibition and/or activating the enzyme (Underwood and Newsholme, 1965; Pogell *et al*. 1968; Behrisch and Hochachka, 1969a, b). The following study was thus initiated to examine the role of ions, substrate and AMP in the regulation of FDPase in the liver of migrating salmon.

RESULTS

Fructose diphosphatase from spawning salmon has a high affinity for substrate (Km = 1.0×10^{-4} M at 15°C) in comparison to rainbow-trout FDPase (Behrisch and Hochachka, 1969a). Affinity of the enzyme for FDP appears to be quite sensitive to temperature; thus, Km of FDP rises about 2 to 3-fold as temperature is increased from 10 to 25° (Table 5.1) with optimum concentrations of Mg^{2+} and Mn^{2+} in the medium. Salmon FDPase is also similar to the trout enzyme in that the FDP saturation curve is sigmoid (n = 2.0) and it is partially inhibited by higher concentrations of FDP (Figs. 5.1 and 5.2). Substrate inhibition is most apparent at high temperature, with Mn^{2+} as the cofactor, in marked contrast to the trout enzyme in which substrate inhibition appears to be independent of temperature, and is more conspicuous in the presence of Mg^{2+}. These results are consistent with those of Underwood and Newsholme (1965) and Taketa and Pogell (1965), who suggest a competitive binding of more than one identically charged substrate molecule to the active site, resulting in an inactive complex. Arrhenius plots of the substrate saturation kinetics show that the rate of reaction is temperature-insensitive between 9 and 15°, a range which corresponds roughly to the habitat temperatures of the organisms used in these experiments. Above and below these temperatures, the slopes of the Arrhenius plots rise sharply (Fig.5.3), with values of Q_{10}

Figure 5.1 Substrate saturation curves of salmon FDPase at a series of temperatures. Assays were carried out at pH 7.4 and in the presence of optimum concentrations of Mn^{2+}. Activity is expressed as mMoles P_i released/l./hr./mg. protein.

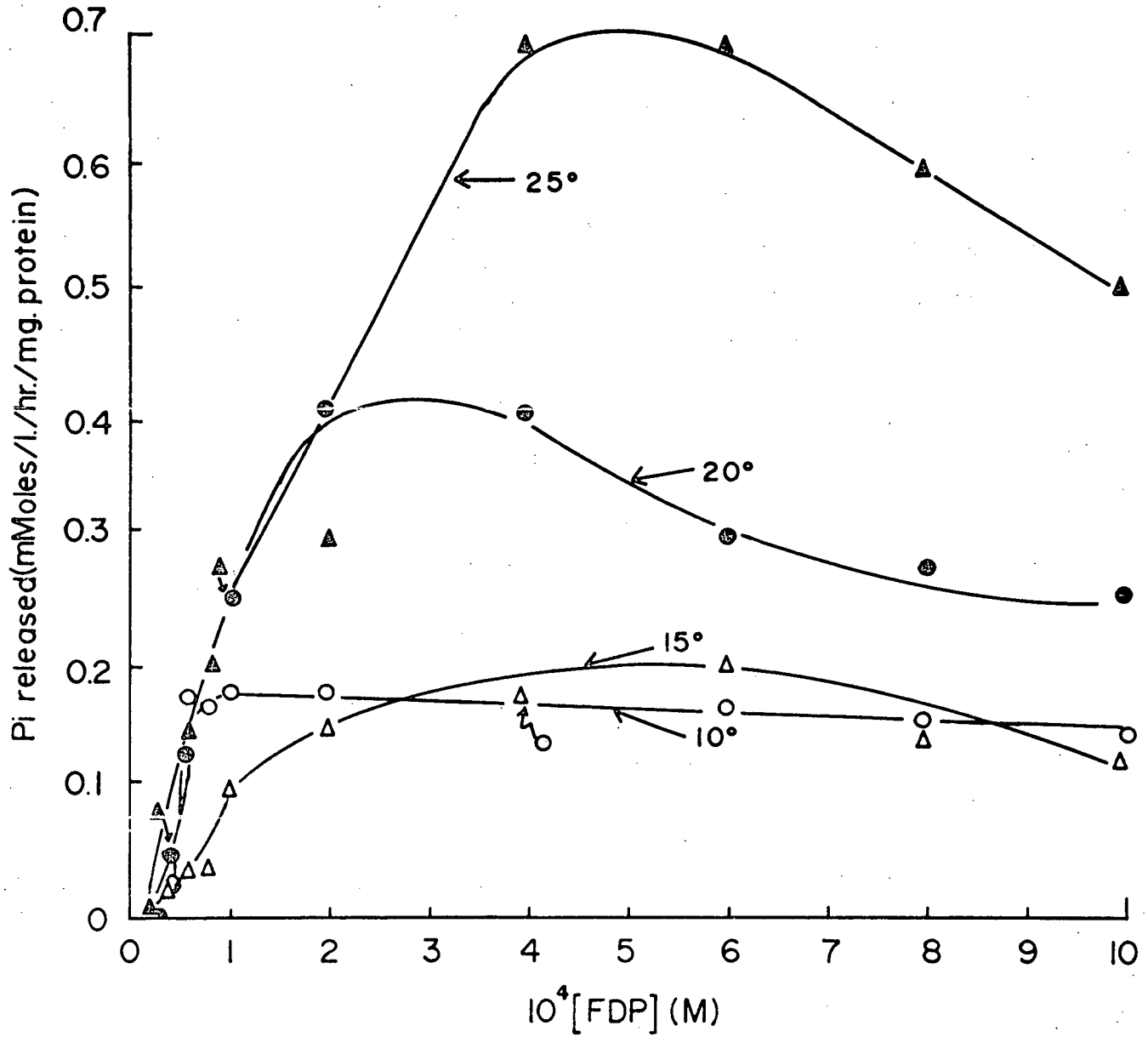

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Pi released(mMoles/l./hr./mg. protein)
25°
20°
15°
10°
0
1
2
3
4
5
6
7
8
9
10
10^4[FDP] (M)

Figure 5.2 Substrate saturation curves of salmon FDPase at a series of temperatures. Assays were carried out in the presence of optimum concentrations of Mg^{2+} and at pH 7.4. Activity is expressed as mMoles P_i released/ l./hr./mg. protein.

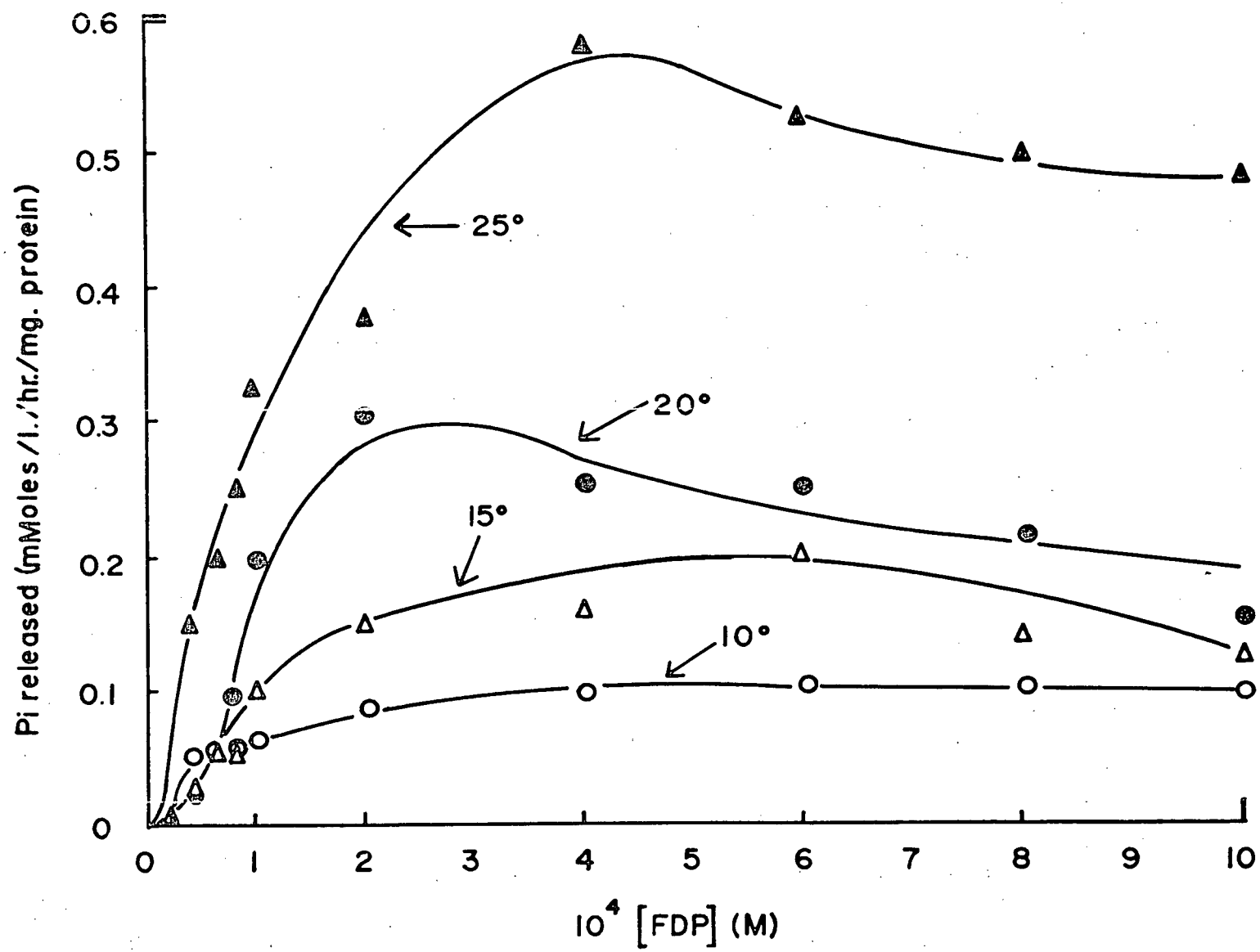

0.6
0.5
0.4
0.3
0.2
0.1
0
Pi released (mMoles/l./hr./mg. protein)
25°
20°
15°
10°
0
1
2
3
4
5
6
7
8
9
10
10⁴ [FDP] (M)

Table 5.I Effect of temperature on affinity and values on n_H of substrate (FDP) of salmon FDPase at optimal concentrations of Mn^{2+} and Mg^{2+}. Assays carried out at pH 7.4.

Temperature	2×10^{-5}M Mn^{2+}			1×10^{-3}M Mg^{2+}		
	Km ($M \times 10^4$)	n_H	Probability of n being zero	Km ($M \times 10^4$)	n_H	Probability of z being zero
10°	0.5	2.37	0.00	0.5	0.87	0.01
15°	1.0	1.74	0.00	1.0	1.47	0.00
20°	8.0	2.90	0.00	9.0	1.52	0.00
25°	17	1.44	0.00	11	1.01	0.00

Table 5.II Activation energies of salmon FDPase for several temperature ranges in the presence of two different cofactors. Ea was calculated from Vopt.

Temperature Range	2×10^{-5} M Mn^{2+} (Ea (Kcal./mole)	1×10^{-3} M Mg^{2+}
0-3°	9.16	115
3-6°	0	0
6-9°	26.3	52.8
9-15°	4.93	0
15-24°	21.7	20.2
24-30°	9.58	9.58

and activation energy rising accordingly (Table 5.II).

Inhibition by AMP: Salmon FDPase is inhibited by AMP (Fig. 5), and the inhibition kinetics are remarkable in two respects. First, there appears to be some activation of the enzyme by low concentrations of AMP after an initial inhibition by AMP at micromolar concentrations, followed by a gradual decline in activity as the concentration of AMP is increased (Fig. 5.4). This anomalous behaviour was observed by Underwood and Newsholme (1965) who were unable to offer an explanation. Second, and most striking, is the relatively high concentration of AMP required to effect 50% inhibition of the reaction at optimal concentrations of FDP and cofactor (Table 5.III). Thus, at all temperatures, the value of K_i of AMP for the salmon enzyme is 3 to 10 times higher than in the trout enzyme, under comparable assay conditions (Behrisch and Hochachka, 1969a). In addition, affinity for AMP does not appear to be very sensitive to temperature between 5 and 15°, a finding which would seem to hold this control feature of the enzyme constant in the species' normal thermal range.

Search for a metabolic activator: A search was made for a metabolic intermediate that would activate salmon liver FDPase and/or reverse the inhibition by AMP. Two metabolites that were considered logical choices were phosphoenolpyruvate and ATP, since the substrate of FDPase, FDP, is a positive modulator of pyruvate kinase (Hess, 1968; Somero and Hochachka, 1968), and ATP is a negative modulator

Figure 5.3 Arrhenius plots for activity of salmon FDPase in presence of optimum concentrations of substrate and Mg^{2+} or Mn^{2+}, and at pH 7.4. Each point was made in duplicate with no variation between duplicates.

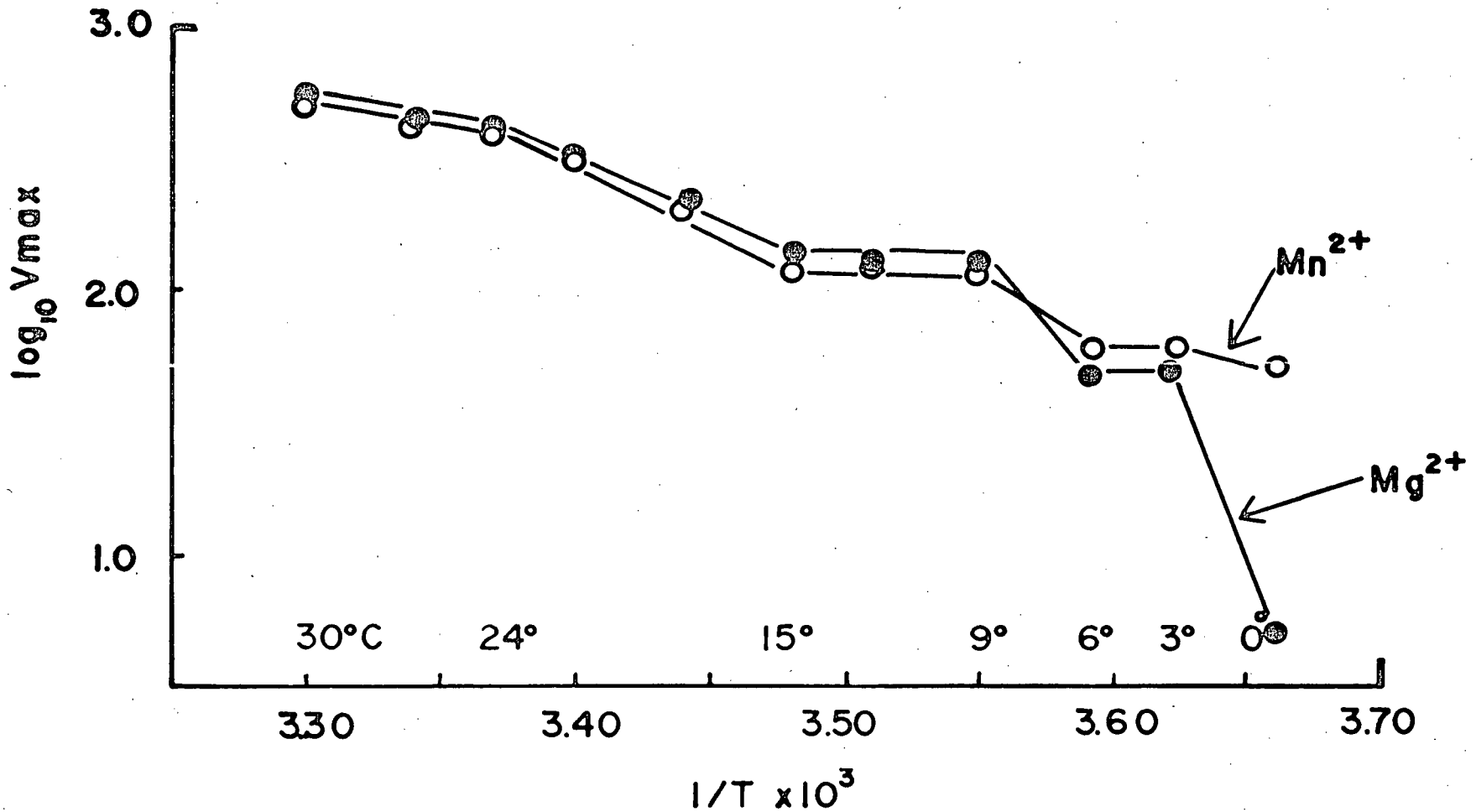
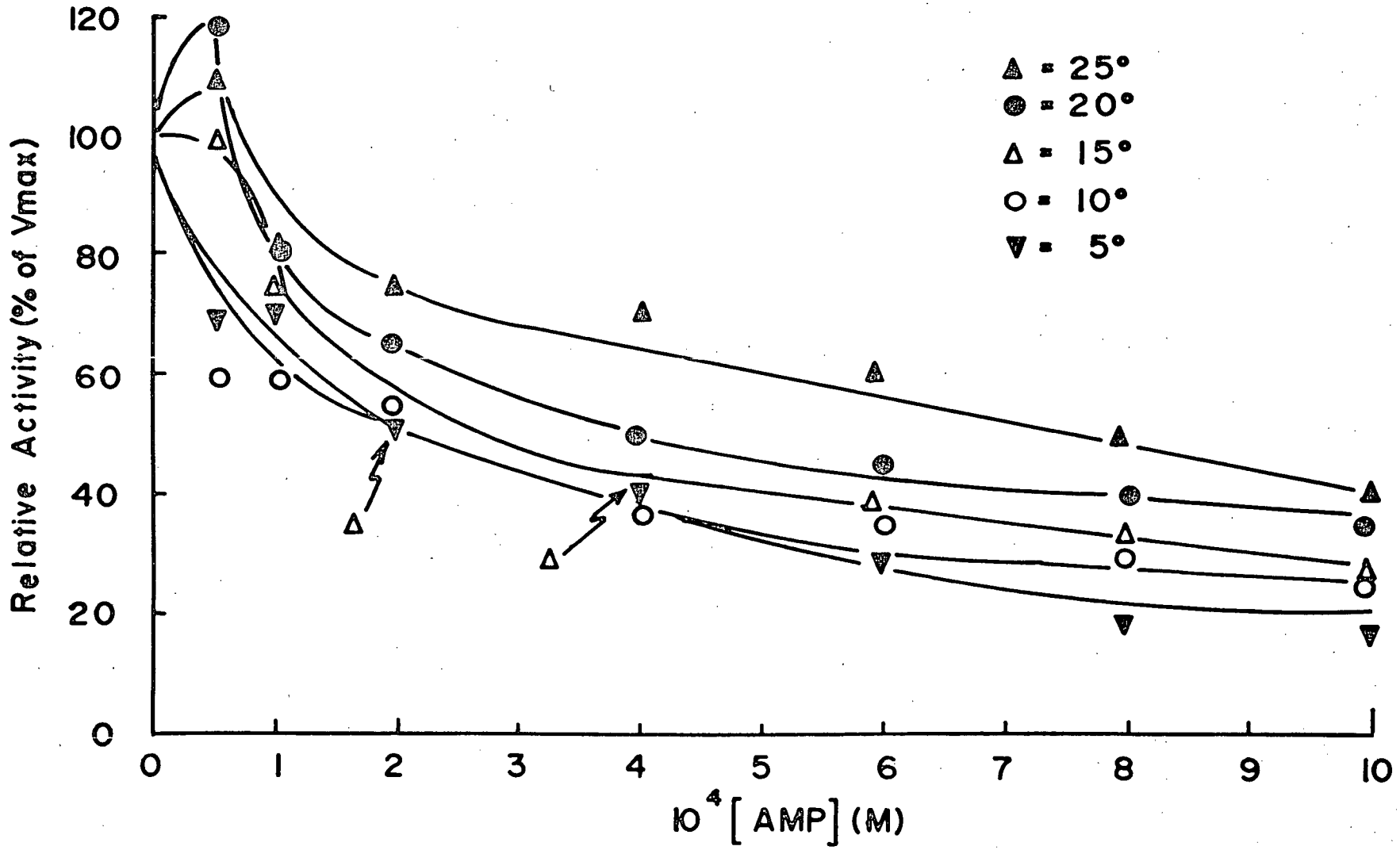

3.0
2.0
1.0
$\log_{10} V_{max}$
Mn^{2+}
Mg^{2+}
30°C
24°
15°
9°
6°
3°
0°
3.30
3.40
3.50
3.60
3.70
$1/T \times 10^3$

Table 5.III AMP inhibition of salmon liver FDPase at a series of temperatures. Assays were carried out in 0.05 TRIS-HCl buffer, pH 7.4, in the presence of 1×10^{-3}M FDP and 2×10^{-5}M Mn^{2+}.

Temperature	K_i of AMP ($M \times 10^4$)
5°	2.0
10°	2.0
15°	3.0
20°	4.0
25°	8.0

Figure 5.4 Inhibition of salmon FDPase by high concentrations of AMP. Assays were carried out in presence of 1 mMolar FDP and 0.02 mMolar Mn^{2+}.


Relative Activity (% of Vmax)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
10^4 [AMP] (M)
▲ = 25°
● = 20°
Δ = 15°
○ = 10°
▼ = 5°

of fish phosphofructokinase (James Freed, personal communication) and mammalian phosphofructokinase (Newsholme and Gevers, 1967). The enzyme was not affected by any of the following metabolites tested: glucose-1-phosphate, glucose-6-phosphate, fructose-6- phosphate, dihydroxyacetone-phosphate, 2-phosphoglycerate, pyruvate, lactate, phosphoneol-pyruvate, ATP, oxaloacetate, citrate, isocitrate, asparagine, glutamate and NADH. Concentrations of ATP higher than 0.1 mM appeared to cause some inactivation of FDPase activity, a phenomenon also observed in swine kidney FDPase (Mendocino *et al*. 1966). A further experiment was attempted in which the pH of the reaction mixture was raised to pH 8.5. The rationale for this was that at alkaline pH, which decreases affinity of the enzyme for AMP (Behrisch and Hochachka, 1969a), the affinity of FDPase for any metabolic activator might be enhanced. However, as at the neutral range, no activation of enzyme activity was seen at high pH.

Determination of energy charge: The total concentrations of tri-, di- and mononucleotides in liver from migrating salmon were determined and the resultant energy charge was calculated. The concentrations of the nucleotides are given in Table 5.IV. Although the assays used are not specific for the adenine nucleotides (Bergmeyer, 1965), the relative concentrations of the total tri-, di- and mononucleotides may be taken to reflect the relative proportions of the adenylates and thus the energy charge of the cell.

It is noteworthy that the mononucleotide concentration

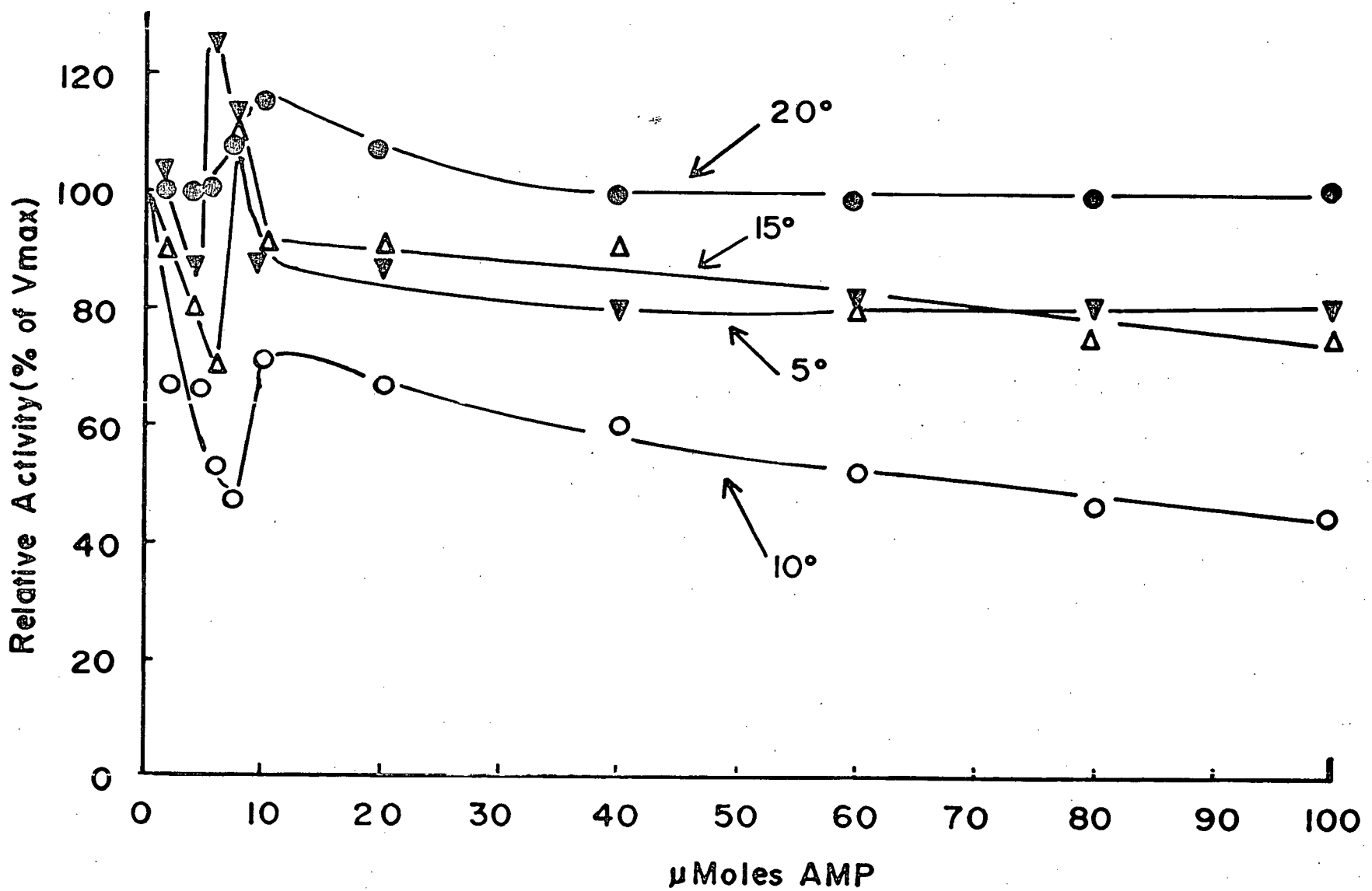
Table 5.IV Concentrations of adenine nucleotides in liver of migrating salmon, and calculation of energy-charge.

Nucleotide	Concentration ($M \times 10^4$)
AMP	5.01
ADP	2.98
ATP	4.14

$$\text{Energy-charge} = \frac{\text{ATP} + \frac{1}{2}\,\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

$$= 0.464$$

Figure 5.5 Inhibition of salmon FDPase by low concentrations of AMP. Assay conditions were as described in Fig. 4.


Relative Activity (% of Vmax)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
μMoles AMP
20°
15°
5°
10°

exceeds that of the trinucleotides. Using the following equation (ATP + ½ADP)/(ATP + ADP + AMP) set forth by Atkinson and Walton (1967), an energy charge of 0.464 was calculated for the salmon liver cell. This value is far below an estimated energy charge of 0.8, below which, under normal circumstances, many biosynthetic reactions would be almost completely inhibited (Atkinson, 1968).

The concentrations of adenylates from liver of migrating salmon are similar to those determined for discharged electroplax from Electrophorus electricus by Williamson et al. (1967b), using comparable assay techniques. Thus, levels of ATP in salmon liver are about one-half those in electric organ, ADP levels are about equal, and concentrations of AMP are two to four-fold higher in migrating salmon liver than in electric organ.

Control by ions: Salmon liver FDPase, like other fish FDPases examined (Behrisch and Hochachka, 1969a, b) shows an absolute requirement for a bivalent cation, which may be fulfilled by either Mg^{2+} or Mn^{2+} (Figs. 5.6 & 5.7). The affinity for Mg^{2+} does not appear to be greatly affected by temperature, although an increase in temperature does cause a slight drop in values of Ka (Table 5.V). The Mg^{2+} saturation curve at pH 7.4 is somewhat unusual in that in addition to the sigmoid saturation curve there is a second inflection which occurs at about 4×10^{-4} M (Fig.5.6). These results are similar to the substrate saturation curves of king crab LDH's and pyruvate kinases (Somero and Hochachka, personal

Figure 5.6 Mg^{2+} saturation curves of salmon FDPase at a series of temperatures. Assays were carried out at pH 7.4 and at a concentration of 1 mM FDP. Activity is expressed as mMoles P_i released/l./hr./mg. protein.

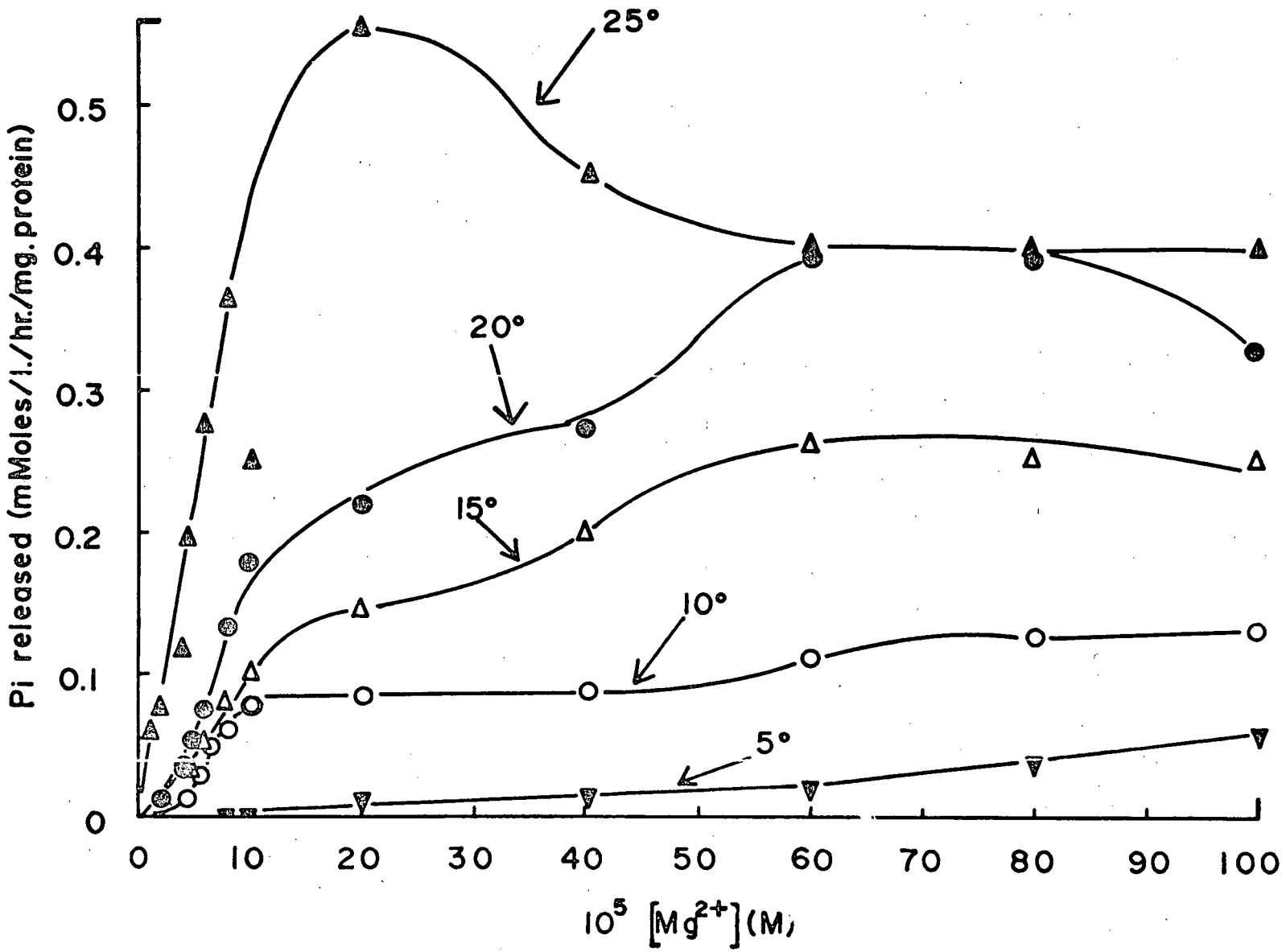

25°
20°
15°
10°
5°
0.5
0.4
0.3
0.2
0.1
0
Pi released (mMoles/l./hr./mg. protein)
0
10
20
30
40
50
60
70
80
90
100
10^5 [Mg^{2+}] (M)

Figure 5.7 Mn^{2+} saturation curves of salmon FDPase at a series of temperatures. Assay conditions were as described in Fig. 6. Activity is expressed as mMoles P_i released/l./hr./mg. protein.

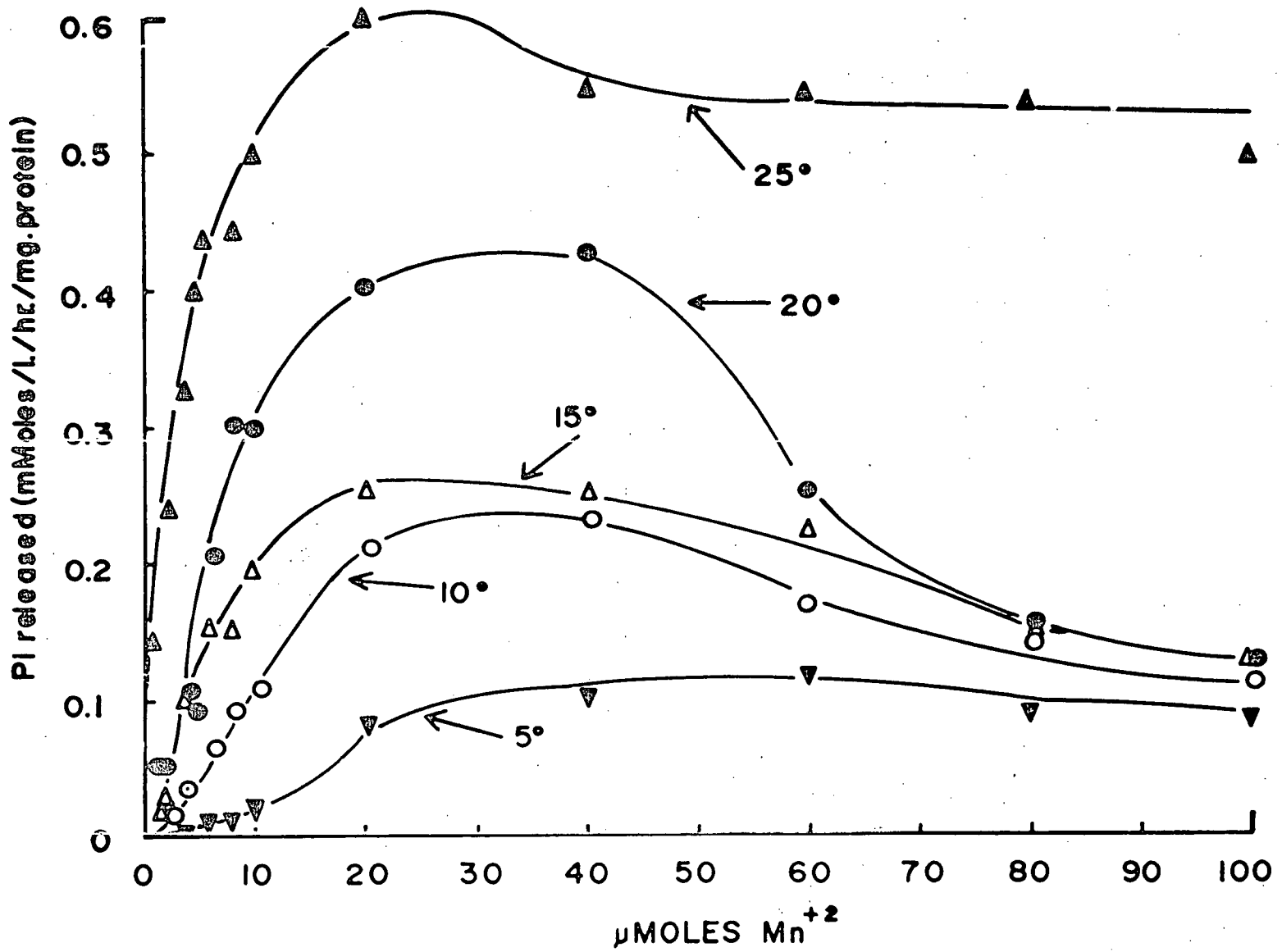

0.6
0.5
0.4
0.3
0.2
0.1
0
Pi released (mMoles/L/hr/mg.protein)
25°
20°
15°
10°
5°
0
10
20
30
40
50
60
70
80
90
100
μMOLES Mn+2

Table 5.V Effect of temperature on affinity and values of n_H of Mn^{2+} and Mg^{2+}. Assays were carried out at pH 7.4 and 1×10^{-3}M FDP.

Temperature	Ka of Mn^{2+} ($M \times 10^6$)	n_H	Probability of n being zero	Ka of Mg^{2+} ($M \times 10^4$)
5°	18	1.94	0.00	no value
10°	11	1.68	0.00	0.8
15°	5.5	0.80	0.06	2.2
20°	5.0	1.65	0.00	1.5
25°	2.5	0.89	0.00	0.8

communication).

The cofactor requirement of the salmon enzyme can also be satisfied by very much lower concentrations of Mn^{2+} (Table 5.V). The Mn^{2+} saturation curve is sigmoid between 5 and 20°, and values of K_a are about 1/40 to 1/10 those for Mg^{2+}. Computerized Hill plots of these results give values of n_H around 2, indicating co-operative interaction between the Mn^{2+} binding sites. At higher temperatures (25°) the Mn^{2+} saturation curve becomes hyperbolic, and values of n_H fall accordingly (Table 5.V). Temperature has a very noticeable effect on the apparent K_a of Mn^{2+}. In the salmon enzyme, a rise in temperature from 5 to 25° results in a 5 to 6-fold increase in affinity of FDPase for Mn^{2+}. The results also show that at low temperatures Mn^{2+} is a much better activator of FDPase activity than is Mg^{2+}. Indeed, it appears that the enzyme is in an inactive conformation at low (0°C) temperatures and with Mg^{2+} in the medium; the enzyme then assumes an active conformation when temperature is raised to 3° (Fig. 5.3). The very large calculated activation energy of 115 Kcal/mole for the temperature range 0 to 3° (Table 5.II) in the presence of Mg^{2+} is in close agreement with enthalpy changes that are recorded during inactivation or extreme conformational changes in protein molecules (Koster and Veeger, 1968). In contrast, when Mn^{2+} is the cofactor, FDPase is almost fully active at low temperature and much less sensitive to temperature

Table 5.VI Effect of varying pH on affinity and values of n_H of Mn^{2+} and Mg^{2+}. Always carried out at 15°C, pH 7.4 and in presence of 1×10^{-3}M FDP.

pH	Ka of Mn^{2+} (M x 10^6)	n_H	Probability of n being zero	Ka of Mg^{2+} (M x 10^5)
7.0	10	1.96	0.00	10
7.5	9	2.02	0.00	14
8.0	4	1.19	0.17	15
8.5	4	1.10	0.09	8
9.0	6	1.25	0.00	8
9.5	6	1.16	0.00	9

changes. Thus it appears that the free Mn^{2+}/Mg^{2+} ratio might be of critical value to the organism in the maintenance of FDPase and gluconeogenic activity.

A rise in pH results in an increase in the affinity of FDPase for Mn^{2+} (Table 5.VI), a result previously reported for both the rainbow trout and lungfish FDPases (Behrisch and Hochachka, 1969a, b). In addition, a rise in pH activates the enzyme dramatically, with an increase from pH 7.5 to pH 9.5 causing a 4-fold increase in $V_{max.}$ at corresponding concentrations of substrate (Fig.5.8). Furthermore, the sigmoidicity of the Mn^{2+} saturation curve at pH 7.5 disappears as pH is raised, and values of n_H fall accordingly (Table 5.VI). The inhibition of FDPase activity at higher concentrations of Mn^{2+} is apparent at all pH's tested (Fig.5.8).

On the other hand, the affinity of the enzyme for Mg^{2+} is not altered appreciably by varying the concentration of H^+ ion (Table 5.VI). The Mg^{2+} saturation curve is sigmoid at low concentrations of cofactor and the second inflection of the curve at around $2 - 4 \times 10^{-4}$ is seen at all pH's (Fig.5.9).

As with other fish FDPases (Behrisch & Hochachka, 1969a, b), there is an alkaline pH optimum (Fig.5.10), which is well beyond the accepted physiological range. The activity-pH curve shows another interesting result: increasing pH activates the enzyme more when Mn^{2+} is the

Figure 5.8 Effect of pH on affinity of salmon FDPase for Mn^{2+}. Assays were carried out at 15°C, and in presence of 1 mMolar FDP. Activity is expressed as mMoles P_i released/l./hr./mg. protein.

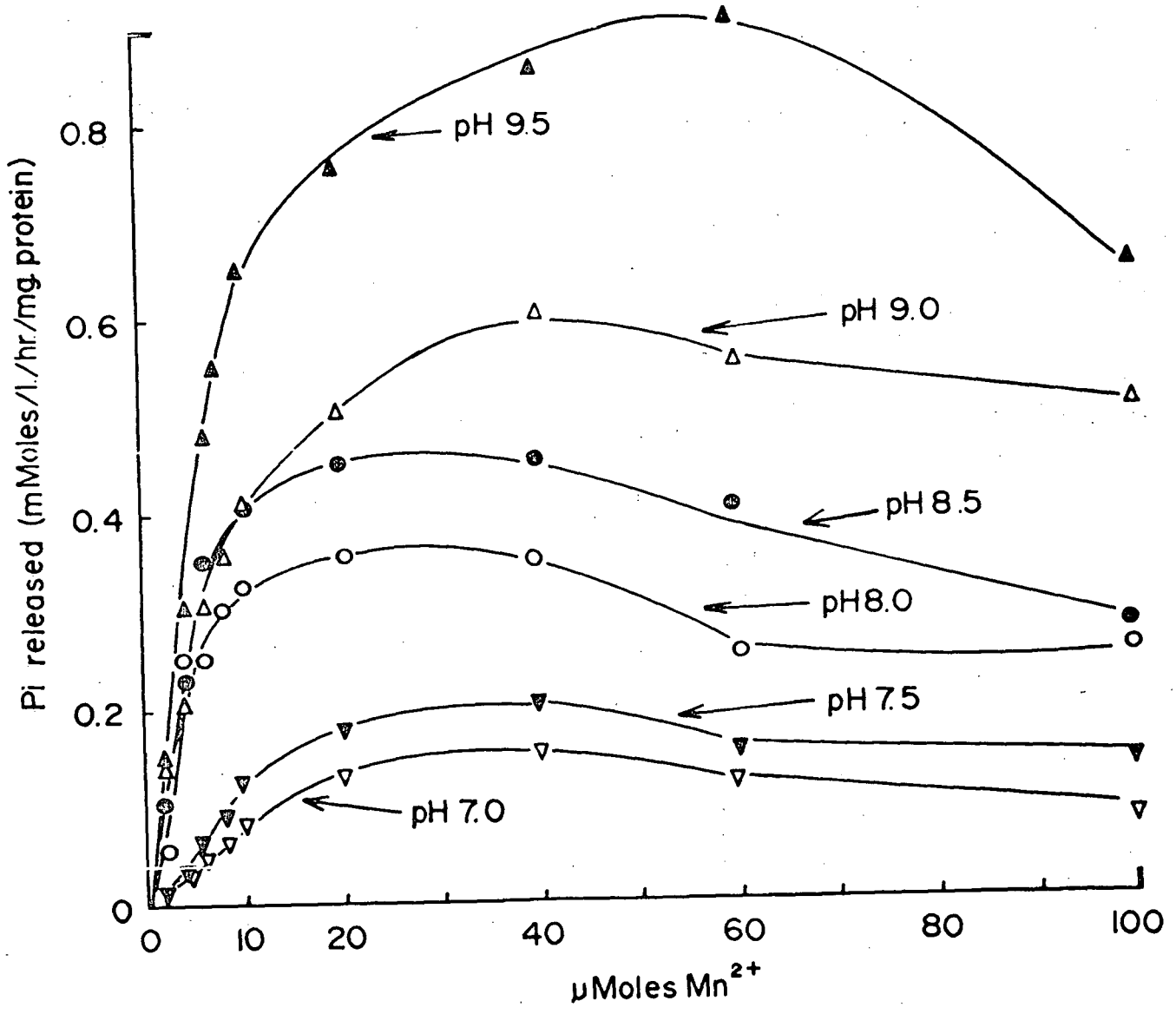

Pi released (mMoles/l./hr./mg. protein)
0.8
0.6
0.4
0.2
0
pH 9.5
pH 9.0
pH 8.5
pH8.0
pH 7.5
pH 7.0
0
10
20
40
60
80
100
μMoles Mn^{2+}

Figure 5.9 Effect of pH on affinity of salmon FDPase for Mg^{2+}. Assay conditions were as described in Fig. 8. Activity is expressed as mMoles P_i released/l./hr./mg. protein.

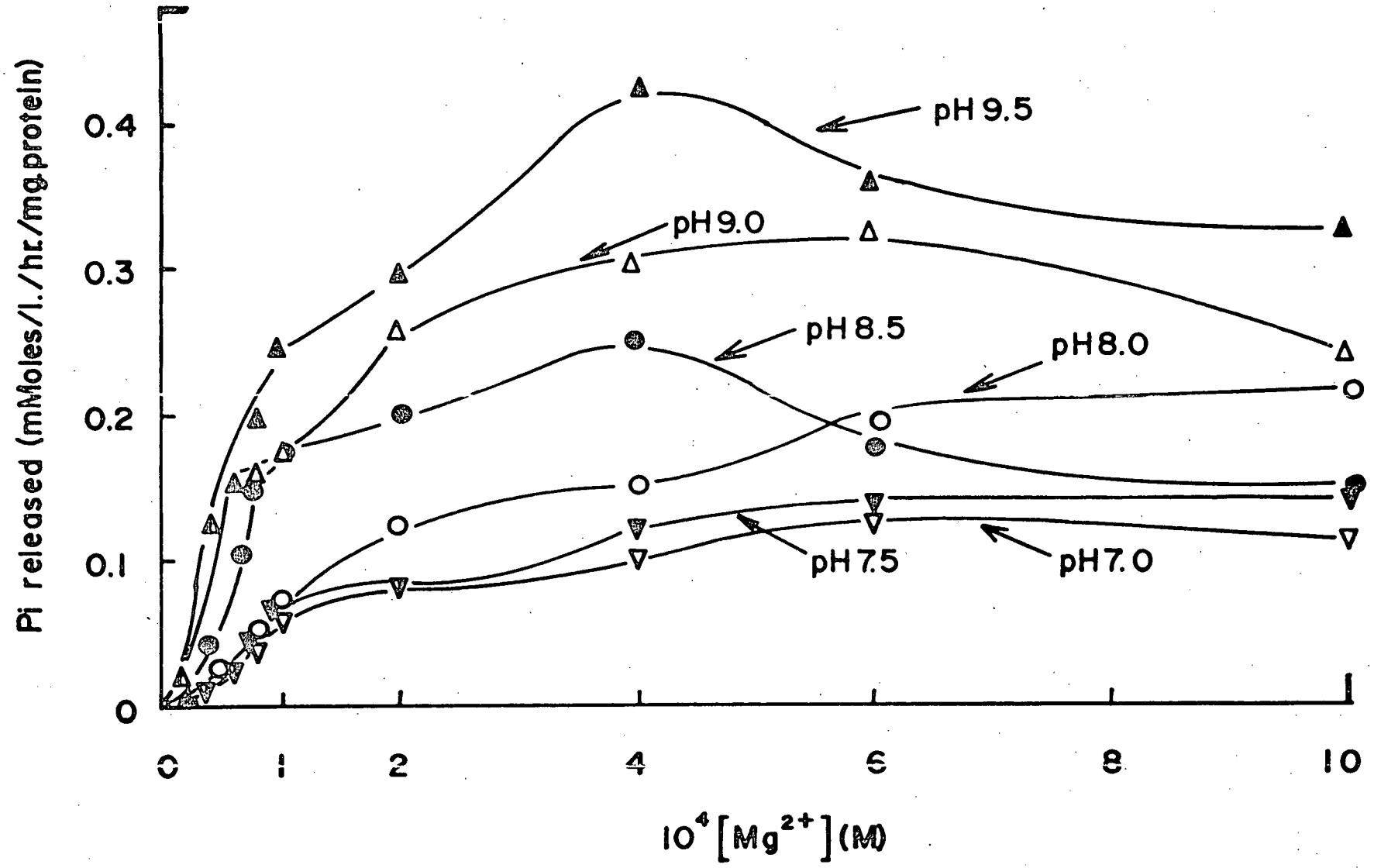
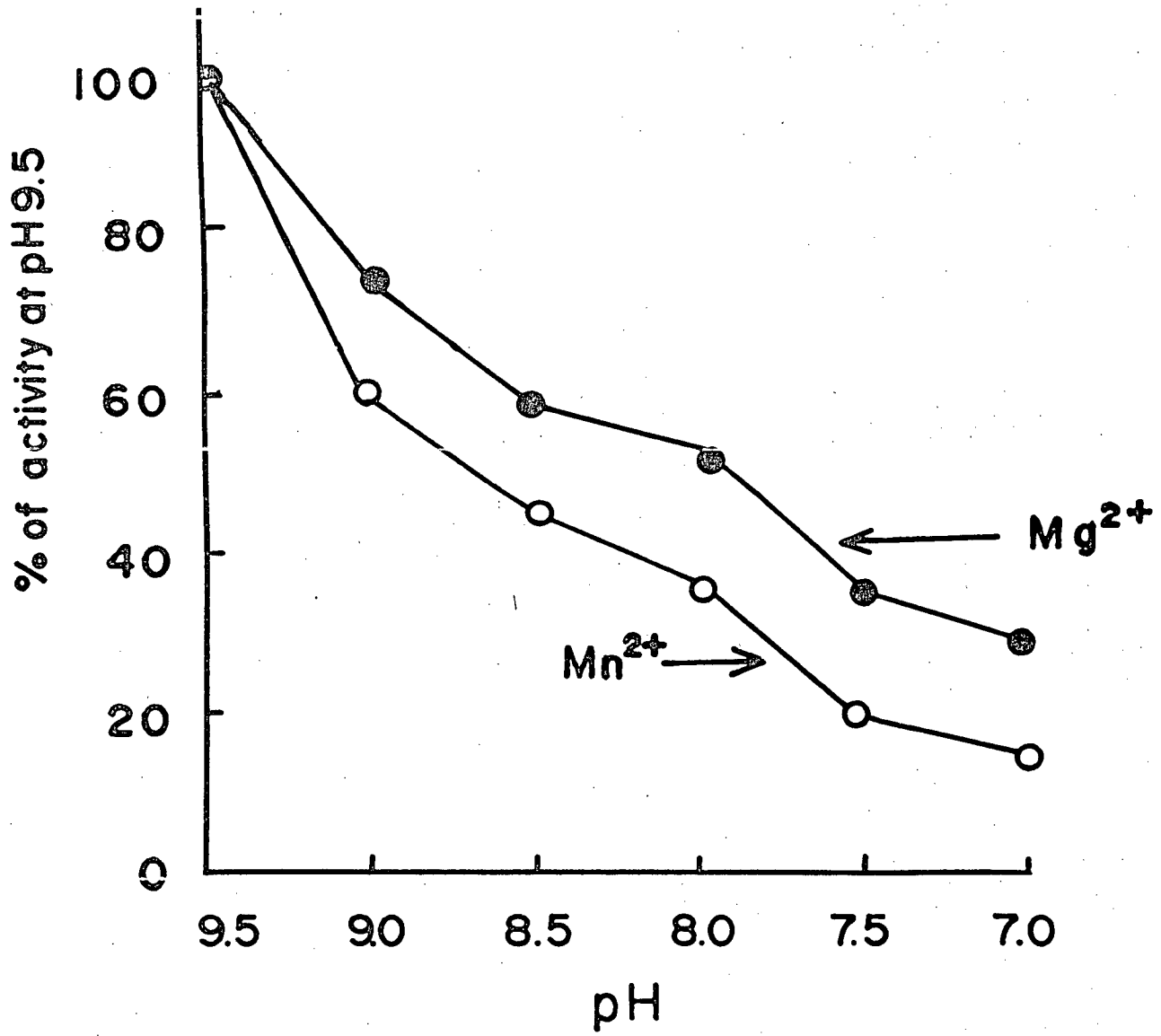

Pi released (mMoles/l./hr./mg.protein)
0.4
0.3
0.2
0.1
0
pH 9.5
pH 9.0
pH 8.5
pH 8.0
pH 7.5
pH 7.0
0
1
2
4
6
8
10
$10^4 [Mg^{2+}]$ (M)

Figure 5.10 Effect of varying pH on activity of salmon FDPase at optimum concentrations of substrate and cofactor. Assays were carried out at 15°C.


100
80
60
40
20
0
% of activity at pH 9.5
9.5
9.0
8.5
8.0
7.5
7.0
pH
Mg2+
Mn2+

cofactor than when Mg^{2+} is present (Fig. 5.10), a result not seen in rainbow trout FDPase.

Calcium ion has been shown to be an effective inhibitor of fish FDPase (Behrisch and Hochachka, 1969a), and this inhibition is competitive with respect to both Mg^{2+} and Mn^{2+}. An increase in pH, which causes an increase in affinity of FDPase for its cofactor, would thus be expected to effect an increase in the affinity for Ca^{2+}. This is in fact seen (Fig.5.11), so that a rise from pH 7.4 to pH 8.5 causes a fall in K_i of Ca^{2+} from 3.9×10^{-4} M to 5.5×10^{-5} M (Table 5.VII) values of K_i which approach the K_a values of Mg^{2+} under identical conditions.

Determination of Ca^{2+}, Mn^{2+} and Mg^{2+} concentrations: The total concentrations of Ca^{2+}, Mn^{2+} and Mg^{2+} in liver from migrating salmon were determined (Coast Eldridge Services, Vancouver, Canada). The concentrations of Ca^{2+} and Mg^{2+} are substantially lower than those reported for mammalian liver; on the other hand, levels of Mn^{2+} are about three to four-fold higher than in mammalian liver (Widdowson and Dickerson, 1964). Since the amount of tissue water is known to change during the spawning run (Idler and Tsuyuki, 1958), and measurements of the concentrations of these ions in the extracellular fluid were not made, the values given in Table 8 cannot yield any precise information concerning intracellular concentrations of free cations. However, measurements of Ca^{2+} (3.5 mM) and Mg^{2+} (.893 mM) from plasma of migrating sockeye salmon (O. nerka) by Idler and

Figure 5.11 Effect of pH on inhibition of salmon FDPase by Ca^{2+}. Assays were carried out at 1 mMolar FDP and 1 mMolar Mg^{2+}.

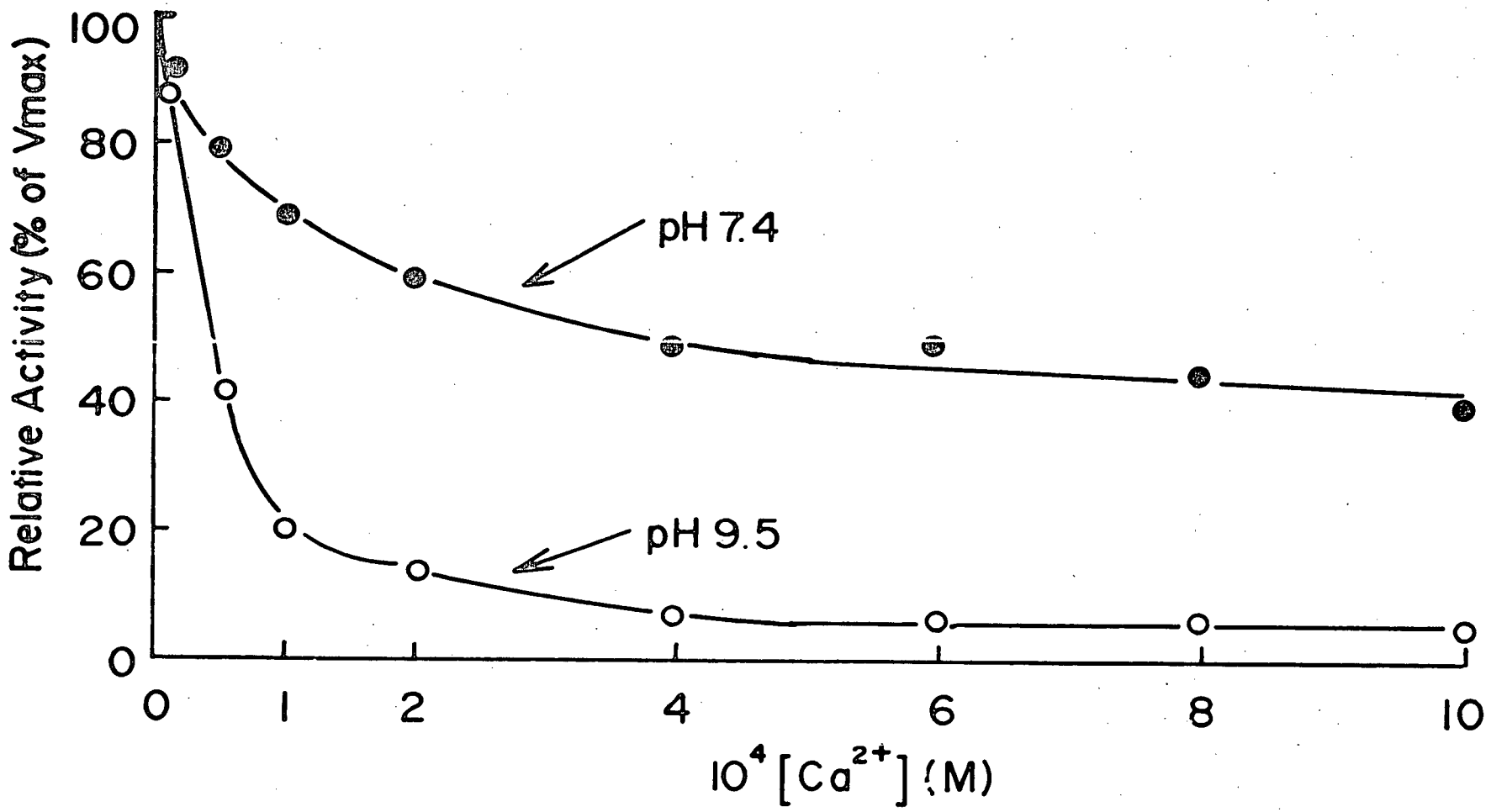

Relative Activity (% of Vmax)
100
80
60
40
20
0
pH 7.4
pH 9.5
0
1
2
4
6
8
10
$10^4 [Ca^{2+}]$ (M)

Table 5.VII Effect of varying pH on K_i of Ca^{2+}. Assays were carried out at 15°C, pH 7.4 and in the presence of 1×10^{-3}M FDP and 1×10^{-3}M Mg^{2+}.

pH	K_i of Ca^{2+} ($M \times 10^5$)
7.4	39.0
8.5	5.5
9.5	5.5

Table 5.VIII Concentrations of calcium, magnesium and manganese ion in liver of migrating salmon.

	in M/Kg tissue	in M/l. tissue water
Ca^{2+}	0.387	0.490
Mg^{2+}	0.213	0.269
Mn^{2+}	0.094	0.119

Tsuyuki (1958), and a ratio of 4:1 for intracellular to extracellular water in Salmo gairdneri (Houston, 1959) make it possible to estimate roughly the intracellular concentrations of Ca^{2+} and Mg^{2+}. Thus, the intracellular concentration of Ca^{2+} appears to be very low, and inhibition of FDPase by this cation would not seem to be significant. The plasma Mg^{2+} concentration in sockeye salmon (0.893 mM) reported by Idler and Tsuyuki (1958) indicates that the intracellular levels of Mg^{2+} and around 0.10 mM, a value very close to K_a of Mg^{2+} at 10° (see Table 5.V). If we assume a distribution of Mn^{2+} similar to that of Mg^{2+}, the estimated intracellular concentration of Mn^{2+} is around 4 to 5×10^{-5} M, a value approximately ten-fold higher than Ka of Mn^{2+} for salmon FDPase at 15°.

DISCUSSION

In our present knowledge of the control of gluconeogenesis, it is generally accepted that the major means of control of FDPase is through negative modulation by AMP (see Atkinson, 1966; Horecker et al. 1966; Newsholme and Gevers, 1967). During prolonged starvation and exercise which occur in the migrating pre-spawning salmon, the energy charge of the liver cell is depressed (Table 3), and since concentrations of AMP are quite high, this condition would tend to maintain FDPase in a "shut-off" conformation. However, under such conditions the maintenance of normal or even high levels of blood glucose would seem to be a condition for survival. Indeed, studies on migrating sockeye salmon indicate that levels of liver glycogen increase by as much as 200% towards the end of the spawning run (Chang and Idler, 1960). Thus, there is a requirement for an active FDPase (Weber et al. 1965). Given such conditions, one might predict mechanisms of activation of FDPase or a means of releasing the enzyme from negative modulation by AMP. Such mechanisms appear to exist in the migrating salmon.

Previous studies (Underwood and Newsholme, 1965; Wallace and Newsholme, 1967; Behrisch and Hochachka, 1969a) have shown that Mg^{2+} can reverse AMP inhibition. However, the changes in Mg^{2+} concentration that would be required are quite high; hence this would not appear to be a plausible

in vivo control feature. Another possible means of regulating FDPase activity involves changes in concentration of H^+ ion (Behrisch and Hochachka, 1969b). As is evident in Figs. 8 and 9, relatively small increases in pH can lead to marked changes in the K_a's of the cofactors as well as dramatic increases in $V_{max.}$. In addition, an increase in pH results in a decrease in affinity of FDPase for AMP (Behrisch and Hochachka, 1969a). Also, similar to the trout and lungfish FDPases, the pH profile of the salmon enzyme in the presence of either Mg^{2+} or Mn^{2+} displays a steep section between pH 7.5 and pH 8.0, a pH range which may be considered physiological in fishes (Steen, 1963). Thus, large changes in activity can be achieved by small changes in pH, a characteristic feature of many regulatory enzymes (Atkinson, 1966). In this connection, an active phosphofructokinase could lead to local acidification, since H^+ is a product of the phosphofructokinase reaction. Conversely, a low rate of glycolysis and phosphofructokinase activity would favour a rise in pH and in this way lead to a significant activation of FDPase.

Furthermore, it is known (Rahn, 1965; Robin, 1962) that the pH of blood from poikilotherms increases with decreasing temperature, and that this increase in pH parallels a rise in the ionization constant (pK_w) of water as temperature falls. The organism must maintain a constant H^+/OH^- ratio at all temperatures (Rahn, 1965) and since pK_w increases with a fall in temperature, an increase in pH is

obtained at lower temperatures. If this requirement for a constant intracellular H^+/OH ratio also confronts the cell, as is suggested by Rahn (1965), it is very probable that intracellular pH also rises with decreasing temperature. This rise in intracellular pH would tend to activate FDPase, and in addition cause a dramatic increase in affinity of fish FDPase for its cationic cofactor (Fig.5.8). Under these conditions, the enzyme would be completely saturated by Mn^{2+} at a concentration of about 10 μ Molar at 10 to 15°. In this connection, Behrisch and Hochachka (1969a) have shown that high pH and/or increased saturation of the enzyme by Mn^{2+} or Mg^{2+} cause a decrease in FDPase-AMP interaction in rainbow trout, and thus prevent or reduce AMP inhibition of FDPase activity. In view of the fact that the habitat temperature of the animals used in these experiments was around 10°, and that a steep section in the pH-activity curve is seen between pH 7.5 and pH 8.0, the temperature-pH relationship supplies a formal mechanism for maintaining FDPase and gluconeogenic activity at low temperature in the migrating salmon.

A third mechanism for control of FDPase activity may involve the substrate. Recent studies (Preiss _et al_. 1967; Behrisch and Hochachka, 1969a, b) indicate that the FDP saturation curve of the enzyme is sigmoidal, suggesting positive cooperative interaction between the substrate binding sites. Thus, since the apparent K_m of FDP appears

to lie well within the physiological concentrations of FDP (Williamson *et al*. 1967a), it appears likely that FDP can serve as a positive modulator as well as substrate of the enzyme.

A fourth mechanism, perhaps the most striking, is the reduced affinity of the salmon FDPase for AMP. While previous studies of FDPase have demonstrated a rather high affinity of fish FDPases for AMP (Behrisch and Hochachka, 1969a, b), this affinity of FDPase for AMP and the temperature sensitivity of this enzyme-modulator interaction are decreased in the salmon. A significant consequence of this reduced sensitivity of AMP-inhibition to temperature is that this control feature of the salmon enzyme remains relatively constant throughout the normal thermal range of the organism.

Thus, there are a number of controls which the liver cell can utilize in the maintenance of FDPase and gluconeogenic activity. A low energy charge in the liver cell would normally favour the concomitant inhibition of gluconeogenesis and activation of glycolysis. However, in the migrating salmon, there is a net gluconeogenesis at a time when the energy-charge of the cell is very low indeed (see Table 4). In every system of which I am aware, selection has favoured the strict regulation of enzymes at control points such as the F-6-P $\rightleftharpoons$ FDP interconversion, so as to prevent the cycling of intermediates and net loss of

energy. Thus, the concomitant inhibition of FDPase and activation of phosphofructokinase by AMP prevents a net hydrolysis of ATP. It is clear that this type of enzyme control is adaptive only under certain physiological conditions, within normal species-specific ranges. In contrast, negative AMP control of FDPase appears to be non-adaptive in an animal like the migrating salmon, which does not feed, is in a state of constant activity, and in which many processes appear to be competing for ATP.

In this connection, migrating salmon synthesize great amounts of cortical steroids (Idler et al. 1959) and carotenoid pigments, and deposit large amounts of carbohydrate in the gonads (Idler et al. 1959). Moreover, Pacific salmon die after spawning; the animal is thus on a "one-way street", and its metabolism must be geared primarily to assure its arrival at the spawning grounds. High levels of blood glucose are an absolute requirement for this, and these can only be supplied when there is an active FDPase. The above controls: high pH, increased concentrations of cationic cofactors, and a reduced affinity to AMP all appear to contribute to maintenance of an active FDPase.

The migrating salmon is also faced with maintenance of control of FDPase and gluconeogenic activity over a wide temperature range. In this connection it is observed that K_m of FDP falls with decreasing temperature (Table 5.I). If the K_m of FDP at physiological temperatures may be taken to

reflect the intracellular concentration range of the metabolite (Srere, 1968), the velocity of the FDPase reaction may actually be higher at low temperatures than at high temperatures (Figs.5.1 & 5.2). Thus, at a concentration of 5×10^{-5} M FDP (K_m of FDP at 10°), the Q_{10} value of the reaction is less than one between 10 and 15°, and about one between 15 and 25° (see Fig.5.1). These results agree with the finding that a decrease in temperature effects a fall in K_m values of substrates for lactate dehydrogenases and pyruvate kinases from a number of fishes (Hochachka and Somero, 1968; Somero and Hochachka, 1968), brain acetylcholinesterase from trout (John Baldwin, personal communication), and fish muscle AMP-aminohydrolase (Makarewicz, 1968; Zydowo et al. 1965).

On the other hand, a rise in temperature causes an increase in affinity of FDPase for its cofactors, so that an increase in temperature from 5 to 25° causes a drop in K_a of Mn^{2+} from 18×10^{-6} M to 2.5×10^{-6} M. This situation is somewhat at odds with the idea of temperature independence of enzyme catalyzed reactions in poikilotherms, since if Mn^{2+} were limiting, an increase in temperature would cause a dramatic increase in enzyme activity. However, the total tissue fluid concentrations of Mn^{2+} and Mg^{2+} (Table 5.VIII) suggest that these ions are always in excess, even if we assume that only a small fraction of the cations exist in the free form. Furthermore, as was mentioned earlier in this

discussion, the pH of the cell probably increases with decreasing temperature (Rahn, 1965), a situation which increases the affinity of FDPase for its cationic cofactor (see also Fig.5.8). A rise in temperature would tend to lower pH, a condition under which affinity of FDPase for Mn^{2+} and Mg^{2+} is reduced. Thus, the observed increase in affinity of trout (Behrisch and Hochachka, 1969a) and salmon FDPase (Fig.5.7) at constant pH for Mn^{2+} and Mg^{2+} can be interpreted as a mechanism to ensure the constant saturation of the FDPase molecule by its cofactor at all temperatures. As mentioned above, when cofactor is saturating, enzyme-FDP affinity increases when the temperature decreases, thus fully compensating for reduced thermal energy. Such changes in enzyme-substrate affinity undoubtedly are coupled with fundamental conformational changes in the enzyme (Iwatsuki and Okazaki, 1967b). These appear to be so large that even at saturating concentrations of substrate, which probably never occur in the cell, there are thermal ranges in which FDPase activity is independent of temperature (Fig.5.3). Thus, with either Mn^{2+} or Mg^{2+} in the medium, Q_{10} values for activity are 1.0 between 9 and 15°, temperatures that correspond well with the habitat temperatures of the organisms used in these investigations. Coupled with the thermal independence of enzyme-AMP affinity, the constant saturation of the enzyme by cofactor, and by increasing enzyme-FDP affinity with decreasing temperature,

this temperature-independence of the enzyme saturated by substrate would seem to allow constant operation of liver FDPase in the migrating salmon, even in fluctuating temperatures.

In this species, as well as in other poikilotherms (Somero and Hochachka, 1968), evolutionary "strategy" appears to be directed towards a gain of independence from the thermal vagaries of the outer environment. This "strategy" seems to have mainly involved a selection for greater affinity for substrate by poikilothermic enzymes at low temperatures, in this way counteracting the effect of reduced thermal energy (see Somero and Hochachka, 1968).

In this connection, a reconsideration of the role of activation energy in poikilothermic systems might be made. In earlier studies on enzymes from poikilotherms, kinetic experiments have been carried out at saturating concentrations of substrates, concentrations which are probably never obtained in the cell. In these studies, the most commonly used measure of "efficiency" is the activation energy, the implication being made that enzymes with lower activation energies would be favoured in environments of low thermal energy. An increasing number of kinetic studies show that such a correlation is not always observed (Hochachka and Somero, 1968). In addition, for a large number of enzymes, including FDPases from trout (Behrisch and Hochachka, 1969a), lungfish FDPase (Behrisch and

Hochachka, 1969b), trout pyruvate kinase (Somero and Hochachka, 1968), and the salmon FDPase (Fig. 5.3) Arrhenius plots are often complex, showing one or more breaks in the curve. It is currently held that such breaks, which often can be affected by substrates or modulators, are due to interconversion of a low temperature form to a high temperature form of the enzyme (Lowry _et al_. 1964; Helmreich and Cori, 1964; Massey _et al_. 1966). Under certain conditions Z-shaped Arrhenius plots may be obtained for amino acid oxidases (Koster and Veeger, 1968) and presumably for other enzymes as well. Available evidence suggests that a steep portion of the Arrhenius curve occurs through a temperature range in which one form of the enzyme is converted to another; in this transition temperature range, the slope probably represents, in addition to the activation energy, the change in activation entropy of the reaction due to conversion of the low temperature conformation to the high temperature one (Koster and Veeger, 1968). Whatever the physical-chemical basis of these effects, it is clear that calculated activation energies can depend strongly upon the temperature range considered as well as upon substrate, cofactor and modulator concentrations (see Fig. 5.3 for e.g.). For these reasons, it appears that in adaptation to unique thermal environments, enzyme "efficiency" as measured by activation energy is not a fundamental site of natural selection (Behrisch and Hochachka, 1969b). Rather,

the sites most sensitive to selection appear to be (1) enzyme affinity for unique substrate (Somero and Hochachka, 1968) and (2) enzyme affinity for cofactors and/or modulators (Walton and Atkinson, 1967; Behrisch and Hochachka, 1969a, b).

CHAPTER 6

SYNOPSIS

In the initial studies of rainbow trout liver FDPase it was observed that the affinity of the enzyme for its allosteric inhibitor (5'AMP) increases dramatically at low temperature, so that at 0°, affinity for AMP is nearly 20 - 30 times greater than at 25°. Since the organism must be capable of maintaining gluconeogenic and hence FDPase activity, it seemed obvious that mechanisms must exist for the activation of FDPase and/or the reversal of AMP inhibition, especially at lower temperatures. Although attempts to identify a metabolic activator of the enzyme were unsuccessful, it became evident that the efficiency of AMP inhibition can be countered by a number of other mechanisms: (1) Increasing cation concentrations, which decrease AMP site-site interactions; (2) Decreasing hydrogen ion concentration, which lowers the K_a of the cationic cofactor, which in turn reduces AMP inhibition and also increases $V_{max.}$ of the reaction; (3) Decreasing the free Mg^{2+}/Mn^{2+} ratios, which effectively decreases the K_a values of the cofactor; (4) Increasing affinity of FDPase for substrate (FDP) and enhancing FDP site-site interaction, particularly at the lower temperatures, and reducing AMP site-site interaction. Furthermore, affinity of trout FDPase for substrate is somewhat enhanced at the lower temperatures, with the result that the reaction rate is independent of temperature at physiological concentrations of substrate.

The lungfish FDPase was somewhat dissimilar to the trout enzyme in that sensitivity to AMP inhibition was reduced. In addition, AMP modulation of enzyme activity was relatively independent of temperature. As in the trout enzyme, affinity for substrate was enhanced at the lower temperatures; thus K_m of FDP was about 7×10^{-5}M at 5° and around 58×10^{-5}M at 35°. This resulted in high enzyme activity at the lower temperatures. However, a rise in temperature from 5 - 35^c caused an almost 5-fold increase in V_{max}. (in presence of optimal amounts of Mn^{2+}, which appeared to stimulate the enzyme much more than did Mg^{2+}). This result is discussed in relation to the finding that gluconeogenesis from pyruvate is favoured in tissue preparations from this animal (Hochachka, 1968). The pH optimum for the lungfish enzyme depends upon the identity of the cofactor present; when Mn^{2+} is in the medium the optimum is shifted towards the neutral range. A relationship is suggested between the effect of H^+ on phosphofructokinase activity and the effect of H^+ on activity of FDPase, since hydrogen ion is a product of the PFK reaction. It is suggested that such a coupling of PFK activity with FDPase activity is analogous to adenylate control of the two enzymes: conditions that favour activity of PFK favour inhibition of FDPase, and conditions that favour activity for PFK favour inhibition of PFK.

In the migrating salmon Oncorhynchus gorbuscha a very low energy charge was calculated for the liver cell. This value was in keeping with the extended starvation and high

degree of muscular and biosynthetic activity in these fish. Affinity of salmon liver FDPase for AMP is approximately 3 to 10 times lower than in the trout, and this interaction is largely independent of temperature. The functional significance of this finding appears to lie in the fact that it permits normal or high rates of FDPase activity under conditions of reduced energy charge. Salmon liver FDPase also exhibits greater affinity for substrate at low temperature, with the result that at low substrate concentrations, reaction velocity is independent of temperature. Indeed, Arrhenius plots of the saturation kinetics show more than several breaks with more than one region in which the reaction rate appears to be independent of temperature. The complexity of the Arrhenius plots suggests a temperature-dependent conversion of more than one form of the enzyme, a conversion which also seems to depend on the identity of the cofactor. Reference was made to the proposed relationship (Rahn, 1965; Reeves and Wilson, 1969; Robin, 1962) between temperature and intracellular pH in poikilotherms. A decrease in temperature results in a rise in pH, which parallels a rise in the ionization constant of water at low temperatures. A rise in pH at low temperature would reduce the thermal sensitivity of the FDPase-AMP interaction, and also maintain catalytic activity independent of temperature changes. Subsequent experiments (to be reported here) on trout FDPase show that this is indeed the case. In a series of experiments at several temperatures, pH was varied in accordance with the observations of Reeves and Wilson

(1969). Thus, at 0°, pH was 8.2, and at 20° the medium was at pH 7.75 (Fig.6.1). Substrate saturation curves under these conditions show that under physiological substrate concentrations, reaction velocity is relatively independent of temperature, with values of Q_{10} ranging from 1.0 between 0 and 10°, and less than 1.0 between 10 and 20°. In a similar experiment (Fig.6.2) the interaction between FDPase and AMP is found to be independent of temperature. Thus values of K_i of AMP are 2×10^{-4} and 2.2×10^{-4}M at 0 and 20°, respectively. Similar results have been obtained for citrate synthetase from rainbow trout liver, in which, under these conditions, the temperature sensitivity of the overall reaction and of the K_i of the inhibitor (ATP) is reduced (Hochachka and Lewis, unpublished observations). In view of the fact that intracellular pH in poikilotherms does appear to change with temperature, this temperature-pH relationship supplies a formal mechanism for maintaining FDPase activity at all temperatures. By these mechanisms, it is proposed that the poikilotherm is able to maintain the activity of FDPase and its overall regulation independent of changes in environment temperature.

Figure 6.1 Substrate saturation curves of rainbow trout FDPase at 0, 10 and 20°. Here pH was varied in accordance with the observations of Reeves and Wilson (1969); thus, at 0°, pH was 8.15, at 10° pH was 7.9 and at mM P_i released/l./hr./mg. protein.

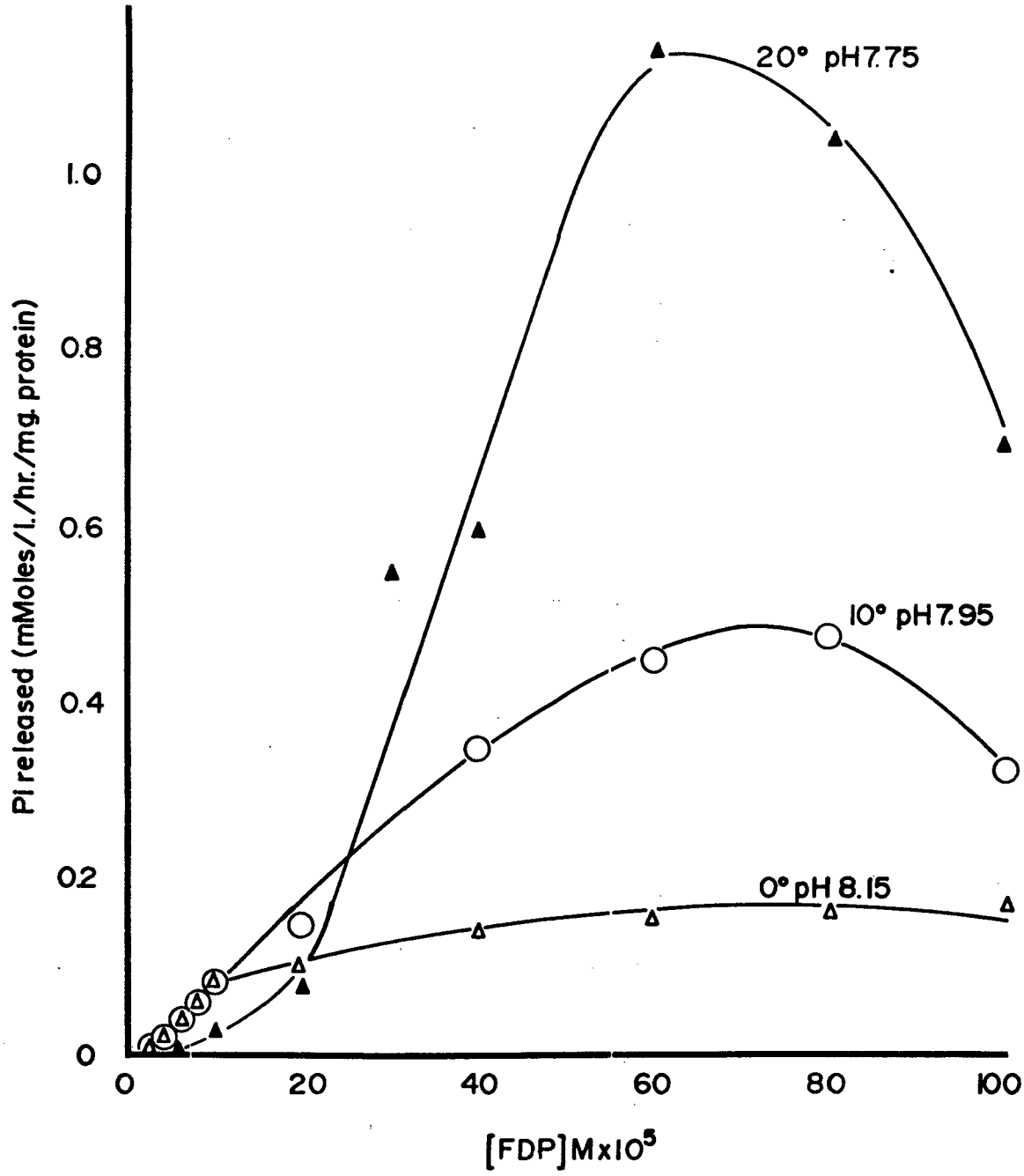
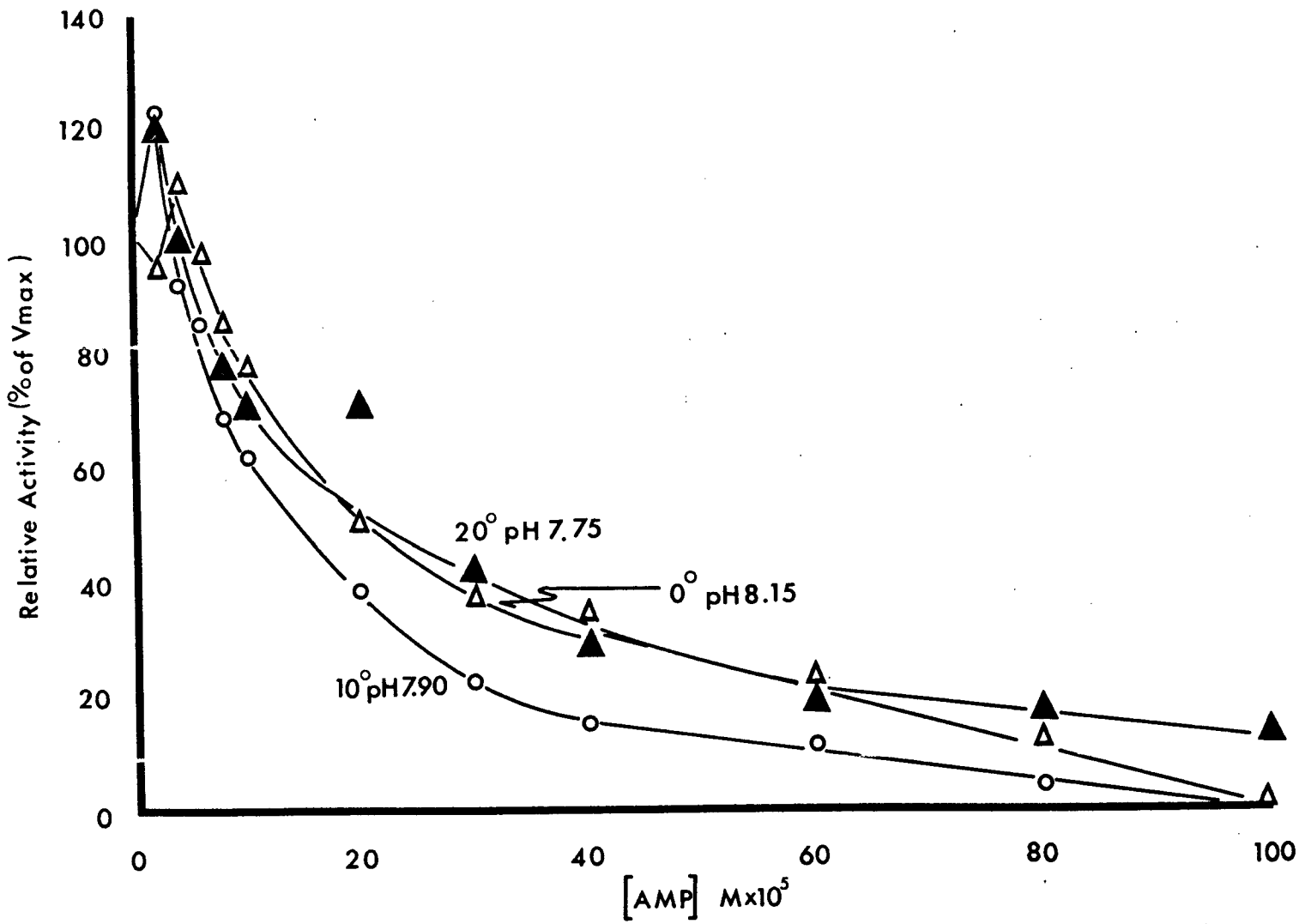

20° pH7.75
10° pH7.95
0° pH 8.15
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Pi released (mMoles/l./hr./mg. protein)
[FDP]Mx10^5

Figure 6.2 AMP inhibition of rainbow trout FDPase under conditions where pH was varied with temperature (see Fig. 1).


140
120
100
80
60
40
20
0
Relative Activity (% of Vmax)
0
20
40
60
80
100
[AMP] Mx10^5
20° pH 7.75
0° pH 8.15
10° pH 7.90

GENERAL DISCUSSION

Two inferences have been made concerning the special properties of regulatory enzymes, mostly on the basis of kinetic, rather than physical and chemical studies: (1) the binding site for the inhibitory end-product is distinct from the active site; (2) the ability of the end-product to act as an inhibitor requires the controlled enzyme to contain subunits. In this connection, Gerhart (1964) presented evidence for the existence of two types of subunits in the enzyme aspartate transcarbamylase; there are two catalytic and four regulatory subunits - a finding later confirmed by Gerhart and Schachman (1968). The most striking outcome of these elegant studies was that the kinetic characteristics of the enzyme were closely dependent upon the state of aggregation of the subunits. The native enzyme displayed sigmoid saturation curves for both substrate and inhibitor, was displaced by pH, and had a high sensitivity to negative and positive modulators. On the other hand, the dissociated enzyme exhibited none of these characteristics; the saturation curves were hyperbolic, effect of pH was markedly reduced, and negative and positive modulators had no effect on enzyme activity. This led to the idea that the subunits interact when they are present in the oligomer (Gerhart, 1964). Subsequently Changeux (1964) suggested that such subunit interactions could be either homotropic (interactions between identical ligands) or heterotropic (interactions

between non-identical stereospecific ligands). Thus it was reasoned that the degree and type of interaction between subunits and/or sites could affect affinity of the enzyme for its ligands as well as activity of the enzyme itself.

The basic kinetic parameter that is modulated during regulation of "allosteric" enzymes is the affinity of the enzyme for its substrate, modulator or cofactor. This is usually expressed as the apparent Km (proportional to the reciprocal of affinity for the ligand in question). Any factor which varies enzyme-ligand affinity will thus effect changes in activity of the reaction. "It is therefore obvious that the operational range of each metabolic regulator is a fundamental control parameter that must be under as strict evolutionary constraint as any other property of the enzyme molecule" (Atkinson, 1966).

It has been observed that temperature can effect structural changes in numerous enzymes (Massey _et al_. 1966; Antonini _et al_. 1966; Metzger _et al_. 1968; Kastenschmidt _et al_. 1968) and that these structural changes are often accompanied by dramatic variations in the degree of interactions between the subunits and the ligand binding sites. Thus, the Hill constant of the homotropic interaction for deoxycytidine 5'monophosphate with deoxycytidylate deaminase at 30° is 4.0, whereas at 10° it is 2.8 (Maley and Maley, 1968a,b). The authors suggested that an explanation for this differential affect might reside in a different configuration of the enzyme at the two temperatures. Evidence that such

conformational changes do occur in the enzyme and that these changes are also very dependent on the identity of the ligand is contained in the finding that the enzyme is converted to a form with a value of $S_{20,w}$ of 6.78 in the presence of dCTP and to one with an $S_{20,w}$ value of 3.63 in the presence of dTTP (Maley and Maley, 1968a). Changeux et al. (1968) and Gerhart and Schachman (1968) presented evidence for different conformational states in aspartate transcarbamylase in the presence and absence of specific ligands. In the presence of carbamyl phosphate and succinate the enzyme exists in a swollen highly reactive form, presumably because of a greater extent of dissociation of the catalytic subunits. When these ligands are absent, the intact enzyme appears to exist in a more compact, slowly reactive conformation, a condition which is favoured by the negative modulator, CTP. In an interpretation of these findings in terms of the Monod, Wyman and Changeux (1965) model, Changeux and Rubin (1968) find that the data fit an enxyme that exists in only two states, one of which binds substrate exclusively and one which binds the feedback inhibitor only preferentially. Furthermore, they concluded that conformational alterations approach completion at concentrations of ligand which only partially saturate the specific binding sites of the enzyme. Such ligand-induced interconversions have also been observed in glycogen synthetase and on the basis of these a model of metabolic control has been proposed at this step (Danforth, 1965). In contrast, a "sequential" model of changes in an

enzyme's conformational state has been suggested by Koshland (1963). This scheme is characterized by the existence of one or more stabilized intermediate conformations of an allosteric enzyme in which all the available sites are not saturated (see also Haber and Koshland, 1967). Support for this theory comes from studies on the allosteric nature of phosphoenol-pyruvate carboxylase from E. coli (Corwin and Fanning, 1968), in which the evidence points to a sequential alteration of the subunits by the substrate, acetyl CoA, rather than an effect in shifting the equilibrium between two symmetrical conformations of the whole enzyme. Whatever the mechanism - and there may be more than one - it is clear that the allosteric behaviour of an enzyme is founded in fundamental changes in its configuration.

In a poikilothermic system, the physical parameter to which subunit-subunit and enzyme-ligand interactions are likely to be most frequently exposed is variations in temperature. A number of large and often differential effects of temperature on enzyme-ligand affinities in poikilotherms have been observed (Ingraham and Maaløe, 1967; Hochachka and Somero, 1968; Somero and Hochachka, 1968; Baldwin and Hochachka, 1969). Available evidence (from homothermic material, to be sure) indicates that these effects of temperature are seated in thermally dependent changes in conformation (Iwatsuki and Okazaki, 1967; Koster and Veeger, 1968). If the transformation between one stable form of the enzyme to the other involved a conformational change and

this process had a high enthalpy of activation such as is commonly found with conformational changes accompanying protein denaturation, then the transformation would be expected to be complete over a narrow range of temperature (Massey et al. 1966). Such a sharp transition would be reflected by one or more sharp breaks in the Arrhenius plot, even though the energies of activation above and below the transition temperature range might not differ greatly. This explanation was also considered likely in the case of fumarase (Massey, 1953) and myosin ATPase (Levy et al. 1959, 1962). Similarly, complex Arrhenius plots have been obtained for trout, lungfish and salmon FDPases (See Chapters 3, 4 and 5). In addition, fish FDPases and a number of other poikilothermic enzymes such as trout acetylcholinesterase (Baldwin and Hochachka, 1969), trout LDH (Hochachka and Somero, 1968), trout pyruvate kinase (Somero and Hochachka, 1968) and king crab phosphofructokinase (James Freed, personal communication) exhibit temperature dependent changes in affinity for their substrates. In the case of fish FDPase, temperature affects enzyme affinity for modulator and cofactor as well.

It is likely that these alterations in enzyme-ligand affinity are rooted in conformational alterations that are caused by changes in temperature. In this connection, Iwatsuki and Okazaki (1967) were able to demonstrate that observed changes in enzyme-substrate affinity were linked to temperature-dependent changes in enzyme configuration.

In the case of fish FDPase, this also seems to be the case. Sucrose density gradient centrifugation of the trout enzyme at two different temperatures (2 and 18°) yields symmetrical peaks, with different values of $S_{20,\ w}$ (Table 6.I). Thus the trout FDPase has an $S_{20,\ w}$ value of around 6.0 at 18° and a value between 6.6 and 7.0 at 2°. Whether these changes in sedimentation velocities are due to differences in the degree of subunit interaction or to variations in tightness of coiling of the individual chains is at present an open question. Further experiments in this direction should yield much useful insight into the matter.

It has been suggested (Noble, 1969) that cooperative allosteric interactions, such as the haeme-haeme interactions in haemoglobin, require that the change in inter-subunit bonding energy accompanying the attachment of a ligand molecule be not uniform but depend rather on the extent to which the protein is already "liganded". Thus the energy of subunit bonding might be the primary cooperative parameter (Noble, 1969). For example, consider the oxygenation of haemoglobin. The ligand reactivity of fully liganded Hb is very similar to that of the liganded isolated chains. Noble then postulates that in unliganded Hb the chains exist in a strained conformation which is characterised by a low affinity for ligand but permits a higher energy of interchain interaction. The interaction of a molecule of ligand raises the free energy of this conformation and thus reduces the interchain bonding energy.

Table 6.I Values of $S_{20,w}$ for rainbow trout FDPase from fish acclimated to 2 and 18°. The enzymes were spun at 39,000 x g for three hours at 2 and 18°.

Acclimation temperature	Values of $S_{20,w}$ Centrifugation temperature	
	2°C	18°C
2°C	6.999	5.885
18°C	6.605	5.815

It seems not unreasonable to accept that the allosteric properties of enzymes confer selective advantage to the cell or organism only when they are effective under physiological concentrations of substrate, modulator and cofactor. Therefore it seems that a parameter governing subunit-subunit interaction might be of importance in the regulation of enzyme activity. It is therefore proposed that a low energy of subunit bonding might be of adaptive advantage to a poikilothermic organism living in an environment of reduced thermal energy. In a poikilothermic enzyme, the binding of ligand at low temperature would result in a great increase in the free energy of the conformation (relative to the proposed low bonding energy). This would lead to enhanced subunit-subunit (or site-site) interaction at low temperature, as observed in the increased Hill constants of substrate saturation in trout FDPase at low temperature (see Chapter 3).

In every case of which I am aware, the regulatory properties of control enzymes are linked to their allosteric behaviour, which in turn is founded on the subunit structure of these enzymes. During adaptation to low temperature environments, selection has obviously favoured the evolution of enzymes whose regulatory characteristics can effectively function in an environment of reduced thermal energy.
It is suggested that two sites at which selective pressure has been greatest during evolution to cold are (1) enhanced enzyme-ligand affinity at low temperatures, and (2) reduced subunit bonding energy, which permits allosteric behaviour

at low temperatures.

In summary, temperature affects the regulatory characteristics of poikilothermic enzymes in various ways. However, at least in the case of fish fructose diphosphatase, the manners in which these control properties respond to thermal changes are evolutionarily designed to maintain the overall regulation of enzyme activity independent of temperature.

GLOSSARY OF TERMS

Cooperativity - the ability of the binding of one molecule of substance (M) to effect a structural change in the enzyme molecule so as to enhance the binding of additional molecules of (M).

Heterotropic - interaction between different ligands.

Homotropic - interactions between identical ligands.

K_m - Michaelis constant; concentration of substrate at which 50% of maximal velocity is obtained. Similarly, use K_i for inhibitor and K_a for activator molecule.

ligand - a molecular group bound to a specific site on the enzyme.

non-equilibrium reaction - a reaction characterized *in vivo* by a massaction ratio smaller than the equilibrium constant of the reaction; physiologically this is an irreversible reaction.

V_{max} - maximum enzyme reaction velocity, similarly, $V_{(opt)}$ indicates optimal reaction velocity.

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