Temperature and the Regulation of Enzyme Activity in Poikilotherms

FRUCTOSE DIPHOSPHATASE FROM MIGRATING SALMON

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(Received 14 July 1969)

1. The calculated energy charge of the liver cell from migrating salmon is very low (0.464), in keeping with the extended starvation and high rates of muscular and biosynthetic activity of these organisms. 2. Affinity of fructose 1,6-diphosphatase for substrate increases with a decrease in temperature. 3. Arrhenius plots of the saturation kinetics are complex and suggest an interconversion of one or more forms of the enzyme; this interconversion is dependent on the identity of the cofactor. 4. Affinity of salmon fructose 1,6-diphosphatase for its allosteric inhibitor (AMP) is lower than in other fructose 1,6-diphosphatases and this enzyme-AMP interaction is largely insensitive to temperature. The functional significance of diminished AMP-sensitivity is that it allows normal or high fructose 1,6-diphosphatase activity during a low energy charge. 5. These findings suggest mechanisms for the maintenance of high rates of gluconeogenesis in salmon during spawning migration.

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 FDPase,† catalysing the conversion:

is essential, since the simultaneous operation of both FDPase and phosphofructokinase, catalysing the conversion:

could lead to a net hydrolysis of ATP and thus to a short-circuit in metabolism (Horecker, Pontremoli, Rosen & Rosen, 1966). In all animal systems considered so far (Newsholme & Gevers, 1967), negative modulation of FDPase by AMP 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 that are migrating upstream to the spawning grounds

do not feed, so that energy is supplied through metabolism of fats and amino acids (Idler, Ronald & Schmidt, 1959; Chang & 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 decreased energy charge (Atkinson & 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.

Secondly, 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 of FDPase by AMP 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 & Pogell, 1965; Hochachka, 1967; Behrisch & 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

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† Abbreviations: FDPase, fructose 1,6-diphosphatase; FDP, fructose 1,6-diphosphate.

enzyme (Underwood & Newsholme, 1965; Pogell, Tanaka & Siddons, 1968; Behrisch & Hochachka, 1969a,b). The present study was thus initiated to examine the role of ions, substrate and AMP in the regulation of FDPase in the liver of migrating salmon.

METHODS AND MATERIALS

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

Experimental methods. Salmon liver homogenate was prepared in 3 parts (v/w) of 10 mM-tris-HCl buffer, pH 7.4, in a Virtis model 23 homogenizer at top speed at 1–2°. The homogenate was spun at about 30000g for 30 min. at 4° and the pellet discarded. The supernatant was brought to 15% saturation with solid (NH₄)₂SO₄ and stirred for at least 3 hr. at 4°. The suspension was then centrifuged, the pellet discarded and the supernatant brought to 55% saturation with solid (NH₄)₂SO₄ and stirred again for at least 3 hr. at 4°. The precipitate was collected by centrifugation at 4° and dissolved in a minimal volume of 10 mM-tris-HCl buffer, pH 7.4. The enzyme solution was then extensively dialysed at 4–5° against 10 mM-tris-HCl buffer, pH 7.4, before use in the assays. This procedure effectively separated FDPase from non-specific alkaline phosphatase, which was assayed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Mo., U.S.A.) as substrate. The reaction medium contained, in 2.0 ml. of 50 mM-tris-HCl buffer of various pH values, various concentrations of FDP (Sigma Chemical Co.), Mg²⁺ or Mn²⁺. The reaction was initiated by the addition of the FDPase preparation and was assayed by measuring P_i release. P_i was determined by the method of Fiske & Subbarow (1925) with the Technicon AutoAnalyzer. Unless otherwise specified, all kinetic experiments were carried out at pH 7.4. Enzyme activity was linear with time for at least 30 min. and was proportional to concentration of enzyme.

Total nucleotide concentrations in the liver cell from spawning salmon were determined by use of ATP and ADP/AMP assay kits (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). These assays are not specific for the adenine nucleotides; the other nucleotides

react quantitatively with the assays (Adam, 1965a,b). However, the relative concentrations of all the tri-, di- and mono-nucleotides 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% (w/v) perchloric acid at 4°. The homogenate was spun at 5000g for 10 min. at 4°, the precipitate discarded and the supernatant used in the nucleotide determinations. Energy charge of the cell was calculated by using the equation of Atkinson & Walton (1967).

RESULTS

FDPase from spawning salmon has a high affinity for substrate (K_m 1.0×10^{-4} M at 15°) in comparison with rainbow trout FDPase (K_m 1.5×10^{-4} M) (Behrisch & Hochachka, 1969a). Affinity of the salmon enzyme for FDP appears to be quite sensitive to temperature; thus K_m for FDP rises about two- to three-fold as temperature is increased from 10° to 25° (Table 1), with optimum concentrations of Mg²⁺ and Mn²⁺ in the medium. It is also similar to the trout enzyme in that the FDP saturation curve is sigmoid ($n_H \approx 2.0$) and is partially inhibited by higher concentrations of FDP (Figs. 1 and 2). Substrate inhibition is most apparent at high temperature, with Mn²⁺ as the cofactor, in marked contrast with the trout enzyme in which substrate inhibition appears to be independent of temperature and is more conspicuous in the presence of Mg²⁺. These results are consistent with those of Underwood & Newsholme (1965) and Taketa & Pogell (1965), who suggested 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 that corresponds roughly to the habitat temperatures of the organisms used in these experiments. Above and below these temperatures, the slopes of the Arrhenius plots increase (Fig. 3), with values of Q_{10} and activation energy rising accordingly (Table 2).

Table 1. *Effect of temperature on affinity and values on n_H of substrate (FDP) of salmon FDPase at optimum concentrations of Mn²⁺ and Mg²⁺*

Assays were carried out at pH 7.4.						
Temp.	0.02 mM-Mn ²⁺			1 mM-Mg ²⁺		
	$10^4 K_m$ (M)	n_H	Probability of n being zero	$10^4 K_m$ (M)	n_H	Probability of n 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

Fig. 1. Substrate saturation curves of salmon FDPase at a series of temperatures: O, 10°; Δ, 15°; ●, 20°; ▲, 25°. Assays were carried out at pH 7.4 in the presence of optimum concentrations of Mn^{2+} . Activity is expressed as m-moles of P_1 released/l./hr./mg. of protein.

Fig. 2. Substrate saturation curves of salmon FDPase at a series of temperatures: O, 10°; Δ, 15°; ●, 20°; ▲, 25°. Assays were carried out at pH 7.4 in the presence of optimum concentrations of Mg^{2+} . Activity is expressed as m-moles of P_1 released/l./hr./mg. of protein.

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. 4). This anomalous behaviour was observed by Underwood & Newsholme (1965), who were unable to offer an explanation. Secondly, and more striking, is the relatively high concentration of AMP required to effect 50% inhibition of the reaction at optimum concentrations of FDP and

Fig. 3. Arrhenius plots for activity of salmon FDPase at pH 7.4 in the presence of optimum concentrations of substrate and Mg^{2+} (●) or Mn^{2+} (O). Each point was made in duplicate with negligible variation between duplicates.

Table 2. Activation energies of salmon FDPase for several temperature ranges in the presence of two different cofactors

Temp. range	E_a was calculated from $V_{opt.}$	
	E_a (kcal./mole)	
	0.02 mm- Mn^{2+}	1 mm- 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

cofactor (Table 3). Thus, at all temperatures, the value of K_i for AMP of the salmon enzyme is three to ten times that of the trout enzyme under comparable assay conditions (Behrisch & Hochachka, 1969a). In addition, affinity for AMP does not appear to be very sensitive to temperature between 5° and 15°, 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 & Hochachka, 1968), and ATP is a negative modulator of fish phosphofructokinase (J. Freed, personal communication) and mammalian phosphofructokinase (Newsholme & Gevers, 1967). The enzyme was not affected by any of the

Fig. 4. Inhibition of salmon FDPase by high concentrations of AMP, at 25° (▲), 20° (●), 15° (△), 10° (○) and 5° (▼). Assays were carried out in the presence of 1 mM-FDP and 0.02 mM-Mn²⁺.

Fig. 5. Inhibition of salmon FDPase by low concentrations of AMP, at 20° (●), 15° (△), 10° (○) and 5° (▼). Assay conditions were as described in Fig. 4.

following metabolites tested: glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, dihydroxyacetone phosphate, 2-phosphoglycerate, pyruvate, lactate, phosphoenolpyruvate, 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 (Mendicino, Beaudreau & Bhattacharyan, 1966). A further experiment was attempted in which the pH of the reaction mixture was increased to 8.5. The rationale for this was that at alkaline pH, which decreases affinity of the enzyme for AMP (Behrisch & Hochachka, 1969a), the affinity of FDPase for any metabolic activator might be enhanced. However, as in the neutral range, no activation of enzyme activity was seen at high pH.

Determination of energy charge. The total concentrations of tri-, di- and mono-nucleotides in liver from migrating salmon were determined and the resultant energy charge was calculated. The mono-

Table 3. AMP inhibition of salmon liver FDPase at a series of temperatures

Assays were carried out in 50 mM-tris-HCl buffer, pH 7.4, in the presence of 1 mM-FDP and 0.02 mM-Mn²⁺.

Temp.	10 ⁴ K _i for AMP (M)
5°	2.0
10	2.0
15	3.0
20	4.0
25	8.0

Table 4. Concentrations of adenine nucleotides in liver of migrating salmon, and calculation of energy charge

Nucleotide	Concentration (mM)
AMP	0.501
ADP	0.298
ATP	0.414

nucleotide concentration exceeded that of the trinucleotides (Table 4). By using the expression $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$ set forth by Atkinson & 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, Herczeg, Coles & Cheung, (1967), using comparable assay techniques. Thus ATP concentrations in salmon liver are about one-half those in electric organ, ADP concentrations are about equal and AMP concentrations 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 & Hochachka, 1969a,b), shows an absolute requirement for a bivalent cation, which may be fulfilled by either Mg²⁺ or Mn²⁺ (Figs. 6 and 7). The affinity for Mg²⁺ does not appear to be greatly affected by temperature, although an increase in temperature does cause a slight decrease in values of K_a (Table 5). The Mg²⁺ saturation curve at pH 7.4 is somewhat unusual in that, in addition to the sigmoid saturation curve, there is a second inflexion, which occurs at about 0.4 mM (Fig. 6). These results are similar to the substrate saturation curves of king-crab lactate dehydrogenases and pyruvate kinases (Somero, 1969).

Table 5. *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 mM-FDP.

Temp.	$10^6 K_a$ of Mn^{2+} (M)	n_H	Probability of n being zero	$10^4 K_a$ of Mg^{2+} (M)
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

Fig. 6. Mg^{2+} saturation curves of salmon FDPase at a series of temperatures: ∇ , 5°; $\circ$, 10°; Δ , 15°; $\bullet$, 20°; $\blacktriangle$, 25°. Assays were carried out at pH 7.4 in the presence of 1 mM-FDP. Activity is expressed as m-moles of P_i released/l./hr./mg. of protein. Fig. 7. Mn^{2+} saturation curves of salmon FDPase at a series of temperatures: ∇ , 5°; $\circ$, 10°; Δ , 15°; $\bullet$, 20°; $\blacktriangle$, 25°. Assay conditions were as described in Fig. 6. Activity is expressed as m-moles of P_i released/l./hr./mg. of protein.

The cofactor requirement of the salmon enzyme can also be satisfied by very much lower concentrations of Mn^{2+} (Table 5). The Mn^{2+} saturation curve is sigmoid between 5° and 20°, and values of K_a are about 40- to ten-fold lower than those for Mg^{2+} . Computerized Hill plots of these results give values

of n_H about 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). 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 five- to six-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 than is Mg^{2+} . Indeed, it appears that the enzyme is in an inactive conformation at low temperatures (0°) and with Mg^{2+} in the medium; the enzyme then assumes an active conformation when temperature is raised to 3° (Fig. 3). The very large calculated activation energy of 115 kcal./mole for the temperature range 0–3° (Table 2) 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 & Veeger, 1968). In contrast, when Mn^{2+} is the cofactor, FDPase is almost fully active at low temperature and much less sensitive to temperature changes. Thus it appears that the free Mn^{2+} /free Mg^{2+} concentration 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 6), a result previously reported for both the rainbow-trout and lungfish FDPases (Behrisch & Hochachka, 1969a,b). In addition, an increase in pH activates the enzyme dramatically, an increase from pH 7.5 to pH 9.5 causing a fourfold increase in V_{max} at corresponding concentrations of substrate (Fig. 8). Further, 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 6). The inhibition of FDPase activity at higher concentrations of Mn^{2+} is apparent at all pH values tested (Fig. 8).

On the other hand, the affinity of the enzyme for Mg^{2+} is not altered appreciably by varying the pH (Table 6). The Mg^{2+} saturation curve is sigmoid at low concentrations of cofactor and the second

Table 6. *Effect of pH on affinity and values of n_H of Mn^{2+} and Mg^{2+}*

Assays were carried out at 15° at pH 7.4 in the presence of 1 mM-FDP.

pH	$10^6 K_a$ of Mn^{2+} (M)	n_H	Probability of n being zero	$10^5 K_a$ of Mg^{2+} (M)
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

Fig. 8. Effect of pH on affinity of salmon FDPase for Mn^{2+} . Assays were carried out at 15° in the presence of 1 mM-FDP. Activity is expressed as m-moles of P_i released/l./hr./mg. of protein. ▽, pH 7.0; ▼, pH 7.5; ○, pH 8.0; ●, pH 8.5; ▲, pH 9.5. Fig. 9. Effect of pH on affinity of salmon FDPase for Mg^{2+} . Assay conditions were as described in Fig. 8. Activity is expressed as m-moles of P_i released/l./hr./mg. of protein. ▽, pH 7.0; ▼, pH 7.5; ○, pH 8.0; ●, pH 8.5; ▲, pH 9.5.

inflexion of the curve at about 0.2–0.4 mM is seen at all pH values (Fig. 9).

As with other fish FDPases (Behrisc & Hochachka, 1969a,b), there is an alkaline pH optimum (Fig. 10), which is well beyond the accepted physiological range.

Ca^{2+} has been shown to be an effective inhibitor of fish FDPase (Behrisc & 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. 11), so that a rise from pH 7.4 to pH 8.5 causes a fall in K_i for Ca^{2+} from 3.9×10^{-4} M to 5.5×10^{-5} M (Table 7), values that approach the K_a values for 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, B.C.,

Fig. 10. Effect of pH on activity of salmon FDPase at optimum concentrations of substrate and Mg^{2+} (●) or Mn^{2+} (○). Assays were carried out at 15°.

Canada). The concentrations of Ca^{2+} and Mg^{2+} are substantially lower than those reported for mammalian liver; on the other hand, Mn^{2+} concentrations are about three- to four-fold higher than in mammalian liver (Widdowson & Dickerson, 1964).

Table 7. *Effect of pH on K_i of Ca^{2+}*

Assays were carried out at 15° at pH 7.4 in the presence of 1 mM-FDP and 1 mM- Mg^{2+} .

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

Fig. 11. Effect of pH on inhibition of salmon FDPase by Ca^{2+} . Assays were carried out in the presence of 1 mM-FDP and 1 mM- Mg^{2+} . ●, pH 7.4; ○, pH 9.5.

Table 8. *Concentrations of Ca^{2+} , Mg^{2+} and Mn^{2+} in liver of migrating salmon*

	Concentration	
	(mole/kg. of tissue)	(mole/l. of tissue water)
Ca^{2+}	0.387	0.490
Mg^{2+}	0.213	0.269
Mn^{2+}	0.094	0.119

Since the amount of tissue water is known to change during the spawning run (Idler & 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 about the intracellular concentrations of free cations. However, measurements of Ca^{2+} (3.5 mM) and Mg^{2+} (0.893 mM) from plasma of migrating sockeye salmon (*Oncorhynchus nerka*) by Idler & Tsuyuki (1958), and a 4:1 ratio 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 & Tsuyuki (1958) indicates that the intracellular Mg^{2+} con-

centrations are about 0.10 mM, a value very close to the K_a for Mg^{2+} at 10° (see Table 5). If we assume a distribution of Mn^{2+} similar to that of Mg^{2+} , the estimated intracellular concentration of Mn^{2+} is about 0.04–0.05 mM, a value about tenfold higher than the K_a for Mn^{2+} of 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 & Gevers, 1967). During the prolonged starvation and exercise that 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 concentrations of blood glucose would seem to be a condition for survival. Indeed, studies on migrating sockeye salmon indicate that liver glycogen content increases by as much as 200% towards the end of the spawning run (Chang & Idler, 1960). Thus there is a requirement for an active FDPase (Weber, Singhal & Srivastava, 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 & Newsholme, 1965; Wallace & Newsholme, 1967; Behrisch & 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, so this would not appear to be a plausible control feature *in vivo*. Another possible means of regulating FDPase activity involves changes in pH (Behrisch & Hochachka, 1969b). As is evident in Figs. 8 and 9, relatively small increases in pH can lead to marked changes in the K_a values for 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 & Hochachka, 1969a). Also, similar to the trout and lungfish FDPases, the pH-activity 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 that 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). 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.

Further, 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 decreasing 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 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. 8). Under these conditions the enzyme would be completely saturated by Mn^{2+} at a concentration of about $10\mu M$ at $10-15^\circ$. Behrisc & 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 decrease AMP inhibition of FDPase activity. Since the habitat temperature of the animals used in these experiments was about 10° , and a steep section in the pH-activity curve is seen between pH 7.5 and pH 8.0, the pH-temperature 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, Biggs & Greenberg, 1967; Behrisc & Hochachka, 1969a,b) indicate that the FDP saturation curve of the enzyme is sigmoidal, suggesting positive co-operative interaction between the substrate-binding sites. Thus, since the apparent K_m for FDP appears to lie well within the physiological concentrations of FDP (Williamson, Cheung, Coles & Herczeg, 1967), 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 decreased affinity of the salmon FDPase for AMP. Although previous studies of FDPase have demonstrated a rather high affinity of fish FDPases for AMP (Behrisc & 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 decreased 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 that 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 fructose 6-phosphate $\rightleftharpoons$ 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 prevent 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 apparently has many processes competing for ATP.

In this connexion, migrating salmon synthesize great amounts of cortical steroids 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 on a 'one-way street', and its metabolism must be geared primarily to assure its arrival at the spawning grounds. High concentrations of blood glucose are an absolute requirement for this, and these can only be supplied when there is an active FDPase. The controls mentioned above, namely high pH, increased concentrations of cationic cofactors and a decreased affinity for 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. It was observed that the K_m for FDP falls with decreasing temperature (Table 1). If the K_m for 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. 1 and 2). Thus, at a concentration of 0.05 mM -FDP (K_m for FDP at 10°), the Q_{10} value of the reaction is less than 1 between 10° and 15° , and about 1 between 15° and 25° (see Fig. 1). These results agree with the finding that a decrease in temperature effects a fall in K_m values for substrates of lactate dehydrogenases and pyruvate kinases from a number of fishes (Hochachka & Somero, 1968; Somero & Hochachka, 1968), brain acetylcholinesterase from trout (J.

Baldwin, personal communication) and fish muscle AMP aminohydrolase (Makarewicz, 1968; Zydowo, Makarewicz, Umiastowski & Purzycka, 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 decrease in K_a for Mn^{2+} from $18 \times 10^{-6}M$ to $2.5 \times 10^{-6}M$. This situation is somewhat at odds with the idea of temperature-independence of enzyme-catalysed 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 8) 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. Further, as already mentioned above, the pH of the cell probably increases with decreasing temperature (Rahn, 1965), a situation that increases the affinity of FDPase for its cationic cofactor (see also Fig. 8). A rise in temperature would tend to lower pH, a condition under which affinity of FDPase for Mn^{2+} and Mg^{2+} is decreased. Thus the observed increase in affinity of trout (Behrlich & Hochachka, 1969a) and salmon FDPases (Fig. 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 decreased thermal energy. Such changes in enzyme-substrate affinity undoubtedly are coupled with fundamental conformational changes in the enzyme (Iwatsuki & Okazaki, 1967). 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. 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 to the habitat temperatures of the organisms used in these investigations. Together with the thermal independence of enzyme-AMP affinity, the constant saturation of the enzyme by cofactor and the 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 & Hochachka, 1968), evolution in thermally unstable environments appears to have resulted in a gain of independence from the thermal vagaries of the outer environment. This adaptation seems to have mainly involved a selection for greater affinity for substrate by poikilothermic

enzymes at low temperatures, to counteract the effect of decreased thermal energy (see Somero & Hochachka, 1968).

In this connexion, 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 that are probably never obtained in the cell. In these studies the most commonly used measure of 'efficiency' is the activation energy, the implication being 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 & Somero, 1968). In addition, for a large number of enzymes, including FDPases from trout (Behrlich & Hochachka, 1969a), lungfish FDPase (Behrlich & Hochachka, 1969b), trout pyruvate kinase (Somero & Hochachka, 1968) and the salmon FDPase (Fig. 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 into a high-temperature form of the enzyme (Lowry, Schulz & Passonneau, 1964; Helmreich & Cori, 1964; Massey, Curti & Ganther, 1966). Under certain conditions, Z-shaped Arrhenius plots may be obtained for amino acid oxidases (Koster & 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 into 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 into the high-temperature one (Koster & Veeger, 1968). Whatever the physical-chemical basis of these effects, it is clear that calculated activation energies can depend strongly on the temperature range considered as well as on substrate, cofactor and modulator concentrations (see Fig. 3 for example). For these reasons, it appears that, in adaptation to unique thermal environments, selection for enzyme 'efficiency' as measured by activation energy is not a fundamental feature of evolution (Behrlich & Hochachka, 1969b). Rather, the sites most sensitive to selection appear to be: (1) enzyme affinity for unique substrate (Somero & Hochachka, 1968) and (2) enzyme affinity for cofactors and/or modulators (Atkinson & Walton, 1967; Behrlich & Hochachka, 1969a,b).

This work was supported by a grant from the Fisheries Research Board of Canada and through a grant from the National Research Council (A-3678) to Dr P. W. Hochachka.

The salmon used in these investigations were collected with the kind co-operation of the scientists of the R/V *Alpha Helix* of the University of California, San Diego, during Cruise C of the Bering Sea Expedition, 1968. It is a pleasure to acknowledge the discussions with Dr Peter Hochachka, Dr George Somero, Mr J. Baldwin and Mr T. Moon which contributed no small amount to the preparation of this manuscript.

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