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Temperature and Enzyme Activity in Poikilotherms

ISOCITRATE DEHYDROGENASES IN RAINBOW-TROUT LIVER

By THOMAS W. MOON and P. W. HOCHACHKA
Department of Zoology, University of British Columbia, Vancouver 8, B.C., Canada

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1. The kinetics of the thermally induced enzyme variants of the supernatant NADP–isocitrate dehydrogenase from rainbow-trout liver are investigated. 2. Fish acclimatized to 2°C (cold-adapted enzyme) and 17°C (warm-adapted enzyme) show different relative distributions of the three NADP–isocitrate dehydrogenase isoenzymes; this has been demonstrated with electrophoresis and electrofocusing techniques. 3. Plots of K_m versus temperature for the cold-adapted and warm-adapted enzyme variants are complex in nature with apparent maximal enzyme–substrate affinity corresponding to the temperature at which the trout is acclimatized. Both substrates, DL-isocitrate and $NADP^+$, give similar curves although the magnitude of the K_m change with temperature is much decreased in the case of $NADP^+$. 4. E_a values of approx. 18kcal/mol were determined for both the cold-adapted and warm-adapted enzyme variants. 5. In an attempt to determine how velocities can be increased at low temperatures, cation, pH requirements, metabolite and enzyme concentrations were examined. 6. NAD–isocitrate dehydrogenase could not be detected in trout tissues.

The relative roles of the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) and the NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) in the metabolism of most organisms are unknown. Largely on the basis of tissue and intracellular distribution, Goebell & Klingenberg (1963, 1964) proposed that only the NAD-linked enzyme was operative in the tricarboxylic acid cycle, and this proposal seems to be supported by recent studies (see, e.g., Nicholls & Garland, 1969). However, in a comprehensive survey of vertebrate muscle types, Crabtree & Newsholme (1970) point out that NADP–isocitrate dehydrogenase activities exceed NAD–isocitrate dehydrogenase activities by some 10–100-fold in mammalian tissues and by up to 300-fold in fishes. During initial phases of this study, we confirmed the observations of Crabtree & Newsholme (1970). We therefore initiated detailed studies of the kinetic properties of the NADP–isocitrate dehydrogenase isoenzymes in fish tissues in order to gain some further insight into the possible functional significance of these enzymes in the metabolism of vertebrate tissues.

In mammals, NADP–isocitrate dehydrogenase occurs in multimolecular forms with electrophoretically distinct supernatant and mitochondrial isoenzymes (Henderson, 1965). Recently the supernatant NADP–isocitrate dehydrogenase has also been reported to occur in isoenzymic forms in bacteria (Reeves, Brehmeyer & Ajl, 1968; Self & Weitzman, 1970), in the carp and in the trout, both of which are thought to be tetraploid (Quiroz-Gutierrez & Ohno, 1970; Wolf, Engel & Faust, 1970).

Previous work from this laboratory (see Hochachka & Somero, 1971) indicate that isoenzymic changes may be correlated with changes in acclimatization temperature and that these changes may be adaptive for the survival of the fish. For this reason, and because NADPH produced by isocitrate dehydrogenase may be utilized during the increased lipogenesis that occurs in fishes at low temperatures (Knipprath & Mead, 1968; Dean, 1969), we were particularly interested in the relationship of this enzyme activity to the acclimatization state of the organism. The results are consistent with the thermal induction of NADP–isocitrate dehydrogenase variants that are well suited for function at their respective acclimatization temperatures. A preliminary report of this work was presented recently (Moon, 1970).

MATERIALS AND METHODS

Experimental animals. Adult rainbow trout (*Salmo gairdneri*) were purchased from Sun Valley Trout Farm, Mission, B.C., Canada. These fish were held at 2°C (cold) or 17°C (warm) for at least 4 weeks under a light regime of 14h light and 10h dark. Each acclimatization tank could maintain 12 fish (approx. 200g each) for this time-period.

These temperatures are consistent with fluctuations seen in many Western Canadian waters.

Preparation of liver supernatant isocitrate dehydrogenase. Livers from a large number of fish were quickly removed and homogenized immediately or frozen (at −30°C) for later use. Tissues were homogenized in 4 vol. of buffer consisting of 100 mM-tris–2 mM-EDTA–2 mM-GSH–0.25 M-sucrose, titrated to pH 7.0 with 1 M-HCl, with an Omni-Mixer (Sorvall) at medium speeds for short time-intervals. This preparation was centrifuged at 40000*g* for 30 min and further at 105000*g* for 1 h. The enzyme was further purified by $(NH_4)_2SO_4$ fractionation between 30 and 60% saturation (all operations were carried out in an ice bath at 0°C). The final extract was resuspended in a minimal quantity of the above buffer, minus sucrose, and dialysed against the same buffer for 2 h to eliminate salts (this procedure tends to decrease the enzyme activity, so it is not recommended to increase this dialysis period). This enzyme preparation is stable for at least 1 month when stored at −30°C.

Assay of isocitrate dehydrogenase activity. Isocitrate dehydrogenase activity was assayed with a Unicam SP. 800 spectrophotometer (Pye Unicam Ltd., Cambridge, Cambs., U.K.) by following the increase in E_{340} due to NADPH formation. The basic reaction mixture contained 100 mM-tris–HCl buffer, pH 8.0 (temperature adjusted according to Sigma Technical Bulletin no. 106B, 1967), 1 mM-$MgCl_2$, 0.15 mM-$NADP^+$, various DL-isocitrate concentrations, and enzyme, added last, in a total volume of 2.0 ml. All chemicals were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. In all cases, isocitrate concentrations are given as DL-isocitrate, even though only 50% of this is the enzymically active *threo*-D_s form (Plaut, 1963). Cuvette temperatures were accurately controlled by the use of a circulating water bath (Lauda Brinkman, K-2/R) coupled to the cuvette holder. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Electrofocusing of isocitrate dehydrogenase. Electrofocusing was carried out according to the method of Haglund (1967) to determine whether the liver isoenzymes had different pI values. The best resolution was attained with a pH 3–10 gradient (LKB Ampholine 8141), run at 300 V for 32 h. Longer electrofocusing runs decreased the enzyme recovery even though the column was maintained at 5°C throughout the run. This poor recovery was most likely a result of protein precipitation within the column.

Electrophoresis of isocitrate dehydrogenase. Horizontal starch-gel electrophoresis was carried out according to Smithies (1955) with a citrate–phosphate buffer system at pH 7.0. The tank buffer was 9 mM-citric acid and 90 mM-Na_2HPO_4; the gel buffer was a 20-fold dilution of this tank buffer. Supernatant samples were applied to three pieces of 5 mM-square Whatman no. 1 filter paper. Adequate separation of isoenzymes was obtained with a 13% starch gel (hydrolysed; Connaught Medical Research Laboratories, Toronto, Ont., Canada) and electrophoresis at 200 V (approx. 20 mA) for 17 h at 4°C. The staining solution contained 62 mg of DL-isocitrate, 15 mg of $NADP^+$, 15 mg of Nitro Blue Tetrazolium, approx. 5 μg of phenazine methosulphate, and 2.5 ml of 20 mM-$MgCl_2$ in 50 ml of 0.1 M-tris–HCl buffer, pH 7.0. All chemicals were obtained from Sigma Chemical Co. Staining was carried out on sliced gels in the dark at room temperature for 2h.

Densitometer traces were taken of a number of sliced gels and the area beneath each peak was determined as a percentage of the total area. A Joyce–Loebl recording microdensitometer was used in all cases.

DEAE-cellulose chromatography. The enzyme was prepared as above, except that the homogenization buffer was 1 mM-tris–1 mM-EDTA–1 mM-β-mercaptoethanol–0.25 M-sucrose, titrated to pH 7.5 with 1 M-HCl. The 30–60%-$(NH_4)_2SO_4$-precipitated fraction was resuspended in a minimal quantity of the above buffer (without sucrose) and dialysed for 12 h against 8 litres of the same buffer. The dialysed enzyme was applied to a column (2.0 cm × 25 cm) of DEAE-cellulose (Whatman, Microgranular) previously equilibrated to 4°C with the same buffer. At least 2 column volumes of the initial buffer were necessary to wash out all non-adhering protein before elution of the NADP–isocitrate dehydrogenase. The enzyme was eluted with a linear gradient in which the tris concentration was increased to 0.3 M. Each 2 ml fraction was collected with a LKB Ultrorac fraction collector and assayed for NADP–isocitrate dehydrogenase activity as above. The gradient was determined by conductivity measurements of eluted samples and compared with those of standard NaCl solutions.

RESULTS AND DISCUSSION

NAD- or NADP-linked isocitrate dehydrogenase. The NAD–isocitrate dehydrogenase does not appear to be present in rainbow-trout tissues. Enzyme activity could not be detected by using the methods of isolation and assay described by Chen & Plaut (1963), nor after attempts at stabilization of the enzyme with substrate, cofactor, cations (Mg^{2+} or Mn^{2+}) or a known positive modulator (AMP). GSH and β-mercaptoethanol were also without effect. Various tissues (liver, gill, brain, heart, and skeletal muscle) known to differ in oxidative potential were examined. In no case was there any significant NAD–isocitrate dehydrogenase activity recorded, although the activity of the NADP-linked isocitrate dehydrogenase enzyme was always relatively high (estimates of NADP–isocitrate dehydrogenase activities are given in Table 1).

Table 1. *Tissue-specific NADP–isocitrate dehydrogenase activity of high-speed* (35000*g*) *supernatant from rainbow trout*

Isolation and assay were as given in the Materials and Methods section. Temperature of assay was 25°C.

Tissue	Enzyme activity (μmol of NADPH/min per g wet wt. of tissue)
Brain	2.86
Liver	4.76
Gill	1.58
Heart	90.50
Skeletal muscle	0.30

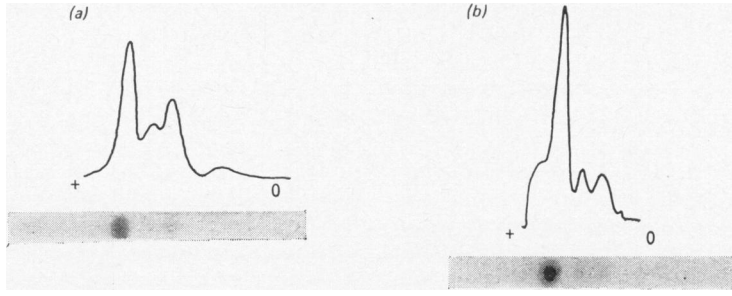

Fig. 1. Starch-gel electrophoretograms of (*a*) the cold-adapted and (*b*) warm-adapted enzyme variants with the corresponding densitometer traces. Electrophoresis was run at 4°C for 17h at 200V with a citrate–phosphate buffer system, pH7.0.

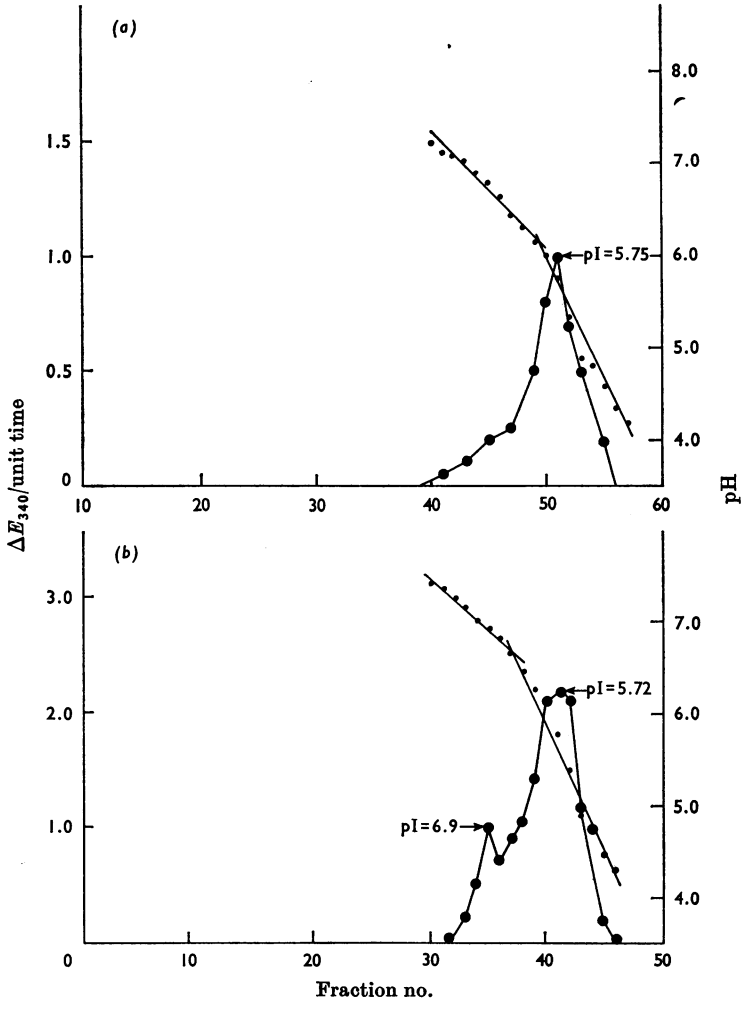

Fig. 2. Concurrent electrofocusing separations on a pH3–10 Ampholyte gradient of (*a*) the cold-adapted and (*b*) the warm-adapted enzyme variants. The gradient was run at 5°C for 32h at 300V. Fractions were assayed at pH8.0.

These results are in close agreement with those of Crabtree & Newsholme (1970), and suggest either that the NAD–isocitrate dehydrogenase does not occur in fish tissues, or that it is highly unstable and its activity in homogenized tissue preparations is no measure of its activity *in vivo*.

Isoenzymes of liver NADP–isocitrate dehydrogenase. In preparations of rainbow-trout liver, three bands of NADP–isocitrate dehydrogenase activity are typically stained in both warm (17°C)- and cold (2°C)-acclimatized trout, but the distribution of these bands is somewhat different. Estimates made from densitometer traces (see Fig. 1) indicate a 5-fold increase in the relative amount of the low-mobility isoenzyme in the cold-acclimatized trout, whereas the warm-adapted enzyme pattern shows approx. 90% of all activity in the fast-moving band. If a system of two subunits, aggregating into three types of dimers is assumed, the results are consistent with two gene loci coding for S-form isocitrate dehydrogenase, a finding in agreement with that proposed for goldfish (Quiroz-Gutierrez & Ohno, 1970) and trout heart M-form isocitrate dehydrogenase (Wolf *et al.* 1970).

The rainbow-trout liver NADP–isocitrate dehydrogenase is also interesting in that the homodimers appear to be stained more intensely than does the heterodimer (see Fig. 1*a*). This suggests either that each band represents more than a single isoenzyme, or that assembly of polypeptide subunits is non-random.

Unlike mammalian NADP–isocitrate dehydrogenase (Henderson, 1965), rainbow-trout liver does not display a unique mitochondrial enzyme form. Similar results have been reported by Wolf *et al.* (1970) for liver from *Salmo irideus*, but not heart, where distinct mitochondrial forms are present. When the enzyme is isolated from carefully prepared trout liver mitochondria, the K_m for substrate is similar to values obtained for supernatant isocitrate dehydrogenase (T. W. Moon, unpublished work). Similar results are available for ox heart NADP–isocitrate dehydrogenase (Higashi, Maruyama, Otani & Sakamoto, 1965). This suggests contamination of the mitochondrial pellet with the S-form isocitrate dehydrogenase.

The results of electrofocusing are consistent with the existence of multiple forms of liver isocitrate dehydrogenase (Fig. 2). However, the recovery of enzyme activity is quite low, so that only shoulders are visible at the pI of the minor enzyme components. The results of DEAE-cellulose chromatography (Fig. 3) are unequivocal in demonstrating three isoenzymic forms in liver of cold-adapted fish. As in starch-gel electrophoresis, the heterodimer (middle peak in Fig. 3) occurs in decreased activity. The major peak in Fig. 3 corresponds to the fast-moving band in Fig. 1. The existence of these isoenzymes of liver NADP–isocitrate dehydrogenase led us to a kinetic study of the cold-adapted and warm-adapted enzyme forms.

Effects of temperature on $V_{max.}$. One method of increasing the catalytic efficiency of enzymes that work at low environmental temperatures is to decrease the energy of activation necessary for the formation of the enzyme–substrate complex. Such an idea has been suggested by Vroman & Brown (1963), although in enzymes examined in this

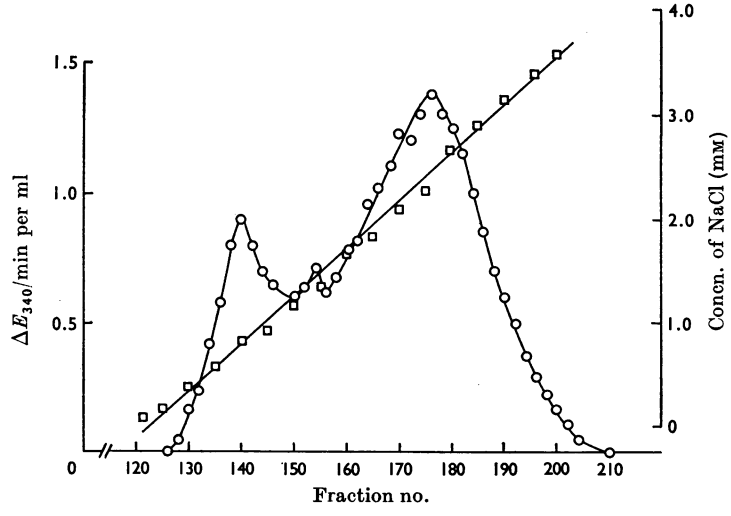

Fig. 3. DEAE-cellulose chromatography of the cold-adpated enzyme variant. See the Materials and Methods section for experimental details. ○, ΔE_{340}; □, NaCl gradient.

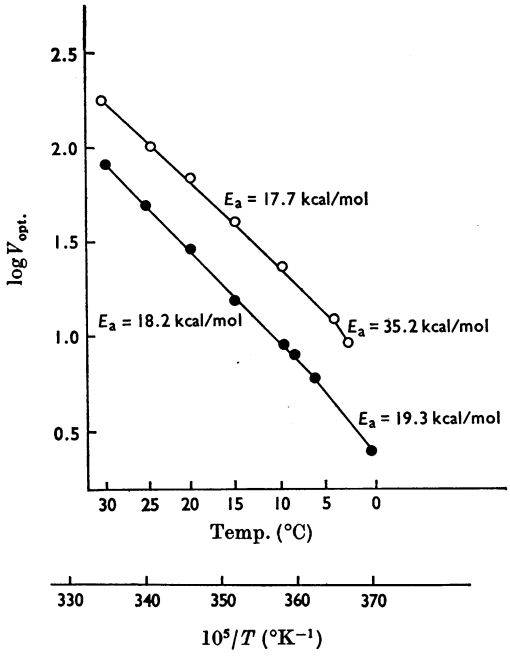

Fig. 4. Arrhenius plots of trout liver NADP–isocitrate dehydrogenase from cold-(●) and warm-(○) acclimatized animals. The assay was carried out at pH 8.0 (200 mM-tris–HCl, temperature adjusted) in the presence of saturating substrate concentrations. E_a was determined from the slope of the line.

laboratory no general relationship between environmental temperature and E_a has been confirmed (Hochachka & Somero, 1971). Arrhenius values (E_a) of 18 kcal/mol for both the warm-adapted and cold-adapted enzyme variants were determined from a plot of $\log V_{opt.}$ versus $1/T$ (Fig. 4).

Effects of temperature on K_m. Fig. 5 shows substrate-saturation curves of DL-isocitrate at different assay temperatures and their Lineweaver–Burk treatments. In the case of the warm-adapted enzyme curves, the activity at 17°C (the temperature at which the fish were acclimatized) is as high as the activity at 25°C when examined at low substrate concentrations (i.e., less than 10 μM-DL-isocitrate). A similar finding is suggested from Fig. 6, which plots $NADP^+$-saturation curves at different temperatures. Again, activity is as high at 15 and 17°C as it is at 25°C. This has been found in a number of enzymes when substrate-saturation curves are plotted at different temperatures, including phosphofructokinase from goldfish (Freed, 1969), fructose diphosphatase from lungfish liver (Behrisch & Hochachka, 1969) and trout brain acetylcholinesterase (Baldwin & Hochachka, 1970).

When the Michaelis constant, K_m, derived from Lineweaver–Burk plots (Figs. 5 and 6) is plotted as a function of assay temperature, complex curves are found (Fig. 7). At the upper biological temperature extreme, K_m varies directly with temperature, approaching a minimal K_m (maximal apparent affinity) within the temperature range at which acclimatization occurs. This is particularly marked for the K_m of DL-isocitrate (Fig. 7), but the relationship is not so extreme in the case of the cofactor, $NADP^+$ (see Fig. 5). Similar complex K_m-versus-temperature curves have been seen for other enzymes from several different tissues of aquatic organisms (Hochachka & Somero, 1971).

The significance of this upper shift in K_m at high biological temperatures has been previously discussed (Baldwin & Hochachka, 1970; Hochachka & Somero, 1971). By increasing the apparent K_m at these high temperatures, the thermal effects on velocity are decreased, thereby leaving the reaction relatively temperature-independent. The upswing seen at the lower biological temperature range for the warm-adapted enzyme (both Fig. 7) is interesting and may be coupled with the high E_a value seen in this region for this enzyme variant (see Fig. 4). Table 2, showing Q_{10} values for both enzymes, demonstrates that for the warm-adapted enzyme variant, between 5 and 10°C, Q_{10} increases with decreasing substrate (DL-isocitrate) concentrations because K_m increases and the thermal energy decreases (Fig. 7). These two effects, high K_m and high Q_{10} (at physiological concentrations), probably set a lower thermal limit for controlled catalytic function by this warm-adapted enzyme variant.

In Fig. 7 it is evident that the minimal K_m values for the two enzyme preparations are similar. In consequence, the rate of catalysis by the cold-adapted enzyme variant at 2°C (minimal K_m for this form) will be much lower than the rate catalysed by the warm-adapted enzyme variant at 17°C (minimal K_m for the warm-adapted form). If acclimatization is to lead to significant rate compensation at any given substrate concentration (Hazel & Prosser, 1970), the K_m of the cold-adapted enzyme variant would have to be much lower than for the warm-adapted form. Since this is not the case, we have considered the possible roles of cations, other metabolites, pH and enzyme concentration in affecting NADP–isocitrate dehydrogenase activity during the thermal acclimatization process.

Effect of temperature on the Michaelis constants of Mg^{2+} and Mn^{2+}. As in the case with NADP–isocitrate dehydrogenase from other organisms (Plaut, 1963), the isoenzymes from trout liver display an absolute requirement for a bivalent cation, which can be fulfilled by either Mg^{2+} or Mn^{2+}. It is evident from Table 3 that the K_a of Mg^{2+} is essentially temperature-independent. Calculated K_a values for Mg^{2+} are in the range $3.3–3.8 \times 10^{-5}$ M for the

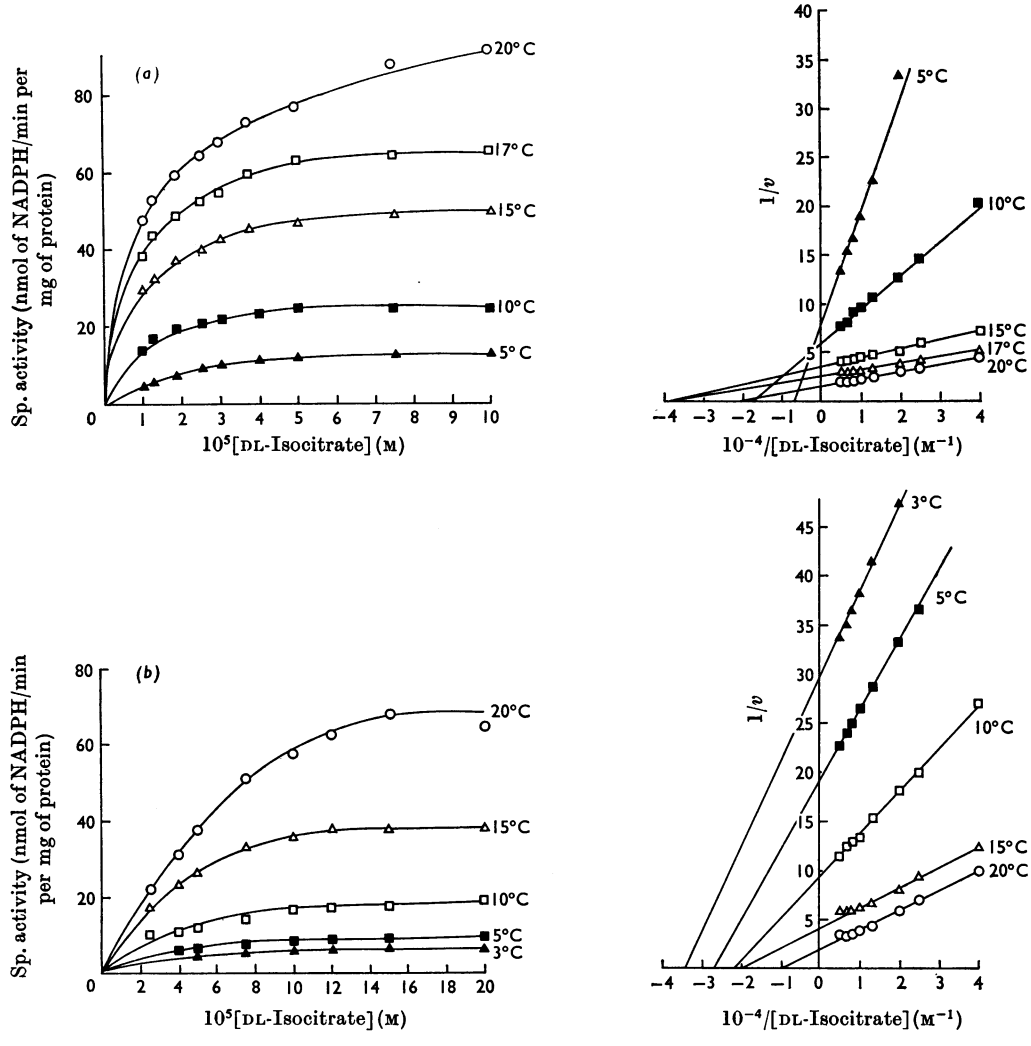

Fig. 5. DL-Isocitrate saturation curves and Lineweaver–Burk plots at different assay temperatures for the (*a*) warm-adapted and (*b*) cold-adapted enzyme variants. Assays were carried out at pH 8.0 (200 mM-tris–HCl, temperature adjusted) with $NADP^+$ and Mg^{2+} saturating.

cold-adapted enzyme and $2.3–2.6 \times 10^{-5}$ M for the warm-adapted enzyme (Table 3). Although the K_a for Mn^{2+} has not been calculated for the warm-adapted form, that for the cold-adapted enzyme is 5-fold smaller than the K_a for Mg^{2+} (see Table 3). Since distribution and concentration of cations is thought to be adjusted during thermal acclimatization in fishes (Hickman, McNabb, Nelson, van Breeman & Comfort, 1964; Houston, Madden & DeWilde, 1970), it is possible that appropriate adjustment in Mg^{2+} or Mn^{2+} concentrations in liver during thermal acclimatization could lead to some compensation of NADP–isocitrate dehydrogenase activity.

Effect of temperature on the pH *optimum.* Fig. 8 is a plot of the relative isocitrate dehydrogenase activity of both enzyme preparations as a function of pH and temperature, and demonstrates typical pH profiles for NADP–isocitrate dehydrogenase (see Higashi *et al.* 1965). In the case of trout liver isocitrate dehydrogenase, both forms display broad pH optima in the pH range 7.0–9.0. An interesting feature of these curves is that as the temperature is decreased (from 25°C to 15°C), the pH optimum for the cold-adapted enzyme is extended towards lower pH values. Work by Rahn (1966) and Reeves & Wilson (1969) has shown that the intracellular and blood pH of fishes is increased as temperature

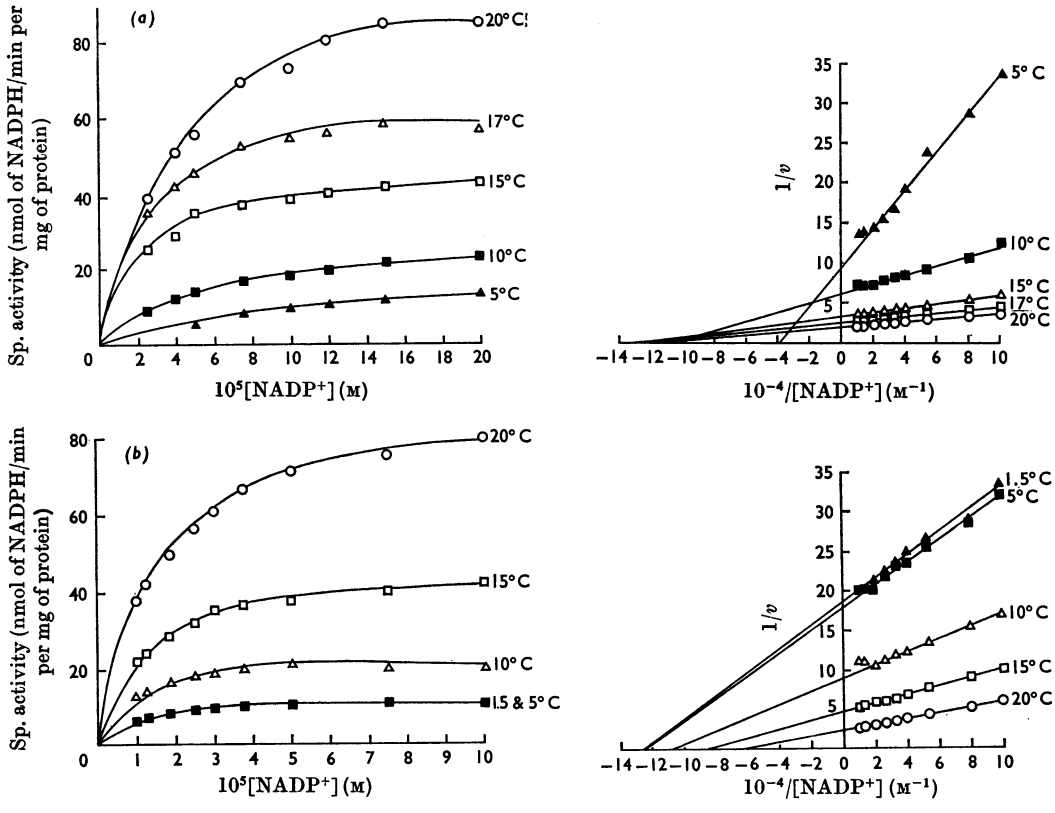

Fig. 6. $NADP^+$ saturation curves and Lineweaver–Burk plots at different assay temperatures for the (*a*) warm-adapted and (*b*) cold-adapted enzyme variants. Assays were carried out at pH 8.0 (200 mM-tris–HCl, temperature adjusted) with DL-isocitrate and Mg^{2+} saturating.

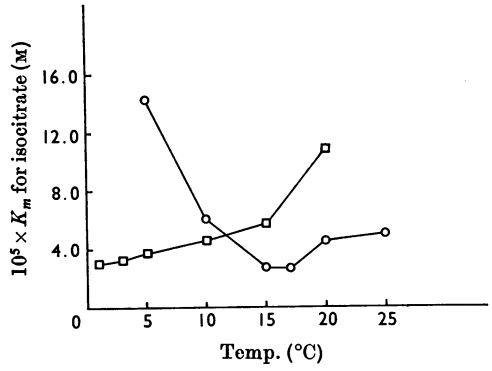

Fig. 7. K_m of DL-isocitrate for the cold-adapted (□) and warm-adapted (○) enzyme preparations as a function of assay temperature. K_m was estimated from the Lineweaver–Burk plots in Fig. 5.

Table 2. *Q_{10} values for the warm-adapted and cold-adapted enzyme variants at various* DL-*isocitrate concentrations*

Experimental details were as given in the text.

Concn. of DL-isocitrate (mM)	Q_{10}, temperature range: 5–10°C, Cold-adapted enzyme variant	5–10°C, Warm-adapted enzyme variant	15–20°C, Cold-adapted enzyme variant	15–20°C, Warm-adapted enzyme variant
1	4.0	5.14	2.92	3.55
0.1	4.0	4.0	2.65	3.47
0.075	3.44	4.0	2.34	3.62
0.05	3.41	7.1	2.0	2.56

decreases. For the cold-adapted form of trout liver isocitrate dehydrogenase, the pH characteristics may exaggerate the effects seen by Rahn and co-workers, thus increasing the catalytic rates at this low temperature. This effect, unlike the K_m–temperature relationship, would thermally stabilize the reaction irrespective of substrate concentrations.

Table 3. *K_a (cations) for the cold-adapted and warm-adapted enzyme variants at various assay temperatures*

Experimental details were as given in the text.

Assay temperature (°C)	K_a (cation): $10^5 \times Mg^{2+}$ concn. (M) Cold-adapted enzyme	$10^5 \times Mg^{2+}$ concn. (M) Warm-adapted enzyme	$10^6 \times Mn^{2+}$ concn. (M) Cold-adapted enzyme
5	3.3	2.5	6.7
10	3.5	2.6	6.4
15	3.3	2.5	—
20	3.8	2.6	6.42
25	3.3	2.3	6.45
30	—	—	6.35

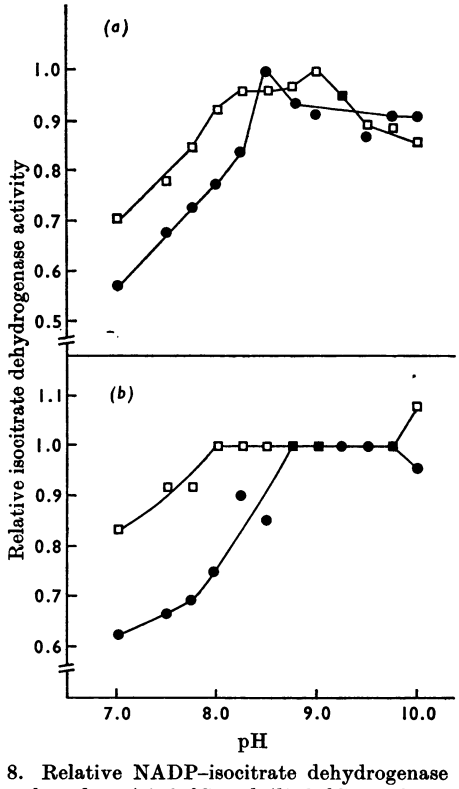

Fig. 8. Relative NADP–isocitrate dehydrogenase activities plotted at (*a*) 25°C and (*b*) 15°C at pH 7–10 for both the cold-adapted (□) and warm-adapted (●) enzyme variants. Assays were carried out with 200 mM-tris–HCl buffer (temperature adjusted) with DL-isocitrate, $NADP^+$ and Mg^{2+} saturating.

Effects of other metabolites. Since a part of the argument favouring NAD–isocitrate dehydrogenase function in the tricarboxylic acid cycle is based on

Table 4. *Effects of various metabolites on the activity of NADP–isocitrate dehydrogenase from rainbow trout liver*

Activity is expressed as percentage of control. Experimental details were as given in the text.

Metabolite	Enzyme activity: 1 mM-DL-Isocitrate	Enzyme activity: 0.1 mM-DL-Isocitrate
Glyoxylate		
(5 mM)	100.0	96.0
(1 mM)	101.0	96.0
(0.5 mM)	104.5	106.0
Oxaloacetate		
(5 mM)	100.0	92.5
(1 mM)	102.5	92.5
(0.5 mM)	104.5	92.5
Glyoxylate (1 mM) + oxaloacetate (1 mM)	42.0	0.0
Citrate		
(5 mM)	100.0	100.0
(1 mM)	100.0	100.0
(0.5 mM)	101.0	100.0
Glutamate		
(5 mM)	102.5	88.5
(1 mM)	100.0	96.0
(0.5 mM)	102.5	106.0
α-Oxoglutarate		
(5 mM)	55.5	0.0
(1 mM)	80.0	34.6
(0.5 mM)	86.5	50.0
Pyruvate		
(5 mM)	91.0	92.5
(1 mM)	91.0	96.0
(0.5 mM)	95.5	92.5
Fructose diphosphate		
(5 mM)	95.5	77.0
(1 mM)	91.0	80.5
(0.5 mM)	82.0	80.5
Phosphoenolpyruvate		
(5 mM)	66.7	57.5
(1 mM)	82.0	88.5
(0.5 mM)	78.0	88.5
NAD^+		
(5 mM)	95.5	115.0
(1 mM)	91.0	100.0
(0.5 mM)	95.5	104.0
NADH		
(0.25 mM)	84.5	81.0
(0.10 mM)	91.0	100.0
(0.05 mM)	91.0	92.5
ATP		
(1 mM)	94.5	62.5
(0.5 mM)	96.5	94.0
ADP		
(5 mM)	100.0	65.5
(1 mM)	101.0	94.0
(0.5 mM)	96.0	100.0
AMP		
(1 mM)	100.0	115.0
(0.5 mM)	94.5	106.0

its responsiveness to regulatory metabolites such as the adenylates (Nicholls & Garland, 1969), we carried out a systematic search for compounds which may serve a regulatory role in the case of trout liver NADP–isocitrate dehydrogenase. Table 4 gives a list of various metabolites which were tested against trout liver NADP–isocitrate dehydrogenase at two different concentrations of substrate. The apparent inhibition of the enzyme by the adenylates, particularly ATP, is due entirely to chelation of Mg^{2+}; at high Mg^{2+} concentrations no positive or negative adenylate effects are seen, unlike the case in some bacteria (Hampton & Hanson, 1969; Parker & Weitzman, 1970) and protozoa (Marr & Weber, 1969*a*,*b*).

However, at high Mg^{2+} concentrations, physiological concentrations of ADP tend to decrease the K_m of DL-isocitrate. Table 5 suggests that at low ADP (0.4mM and less), the K_m of the cold-adapted enzyme variant of 10°C is decreased up to 3-fold, whereas higher concentrations are not as effective. Such an effect is known for the NAD–isocitrate dehydrogenase from animal tissues (Chen & Plaut, 1963), but has not been reported for the NADP-linked enzyme.

Also, Fig. 9 demonstrates that for the cold-enzyme variant, α-oxoglutarate is a competitive inhibitor of NADP–isocitrate dehydrogenase, increasing the K_m for DL-isocitrate by a factor of 2.5 at 1mM- and 10 at 5mM-α-oxoglutarate. These concentrations of α-oxoglutarate appear to be in the physiological range (Williamson, Scholz &

Table 5. *Effects of ADP on the K_m of* DL-*isocitrate in the cold-adapted enzyme variant assayed at* 10°C

Experimental details were as given in the text.

Concn. of ADP (mM)	$10^5 \times K_m$ [DL-isocitrate] (M)
0	4.8
0.1	2.8
0.4	1.57
0.8	2.58

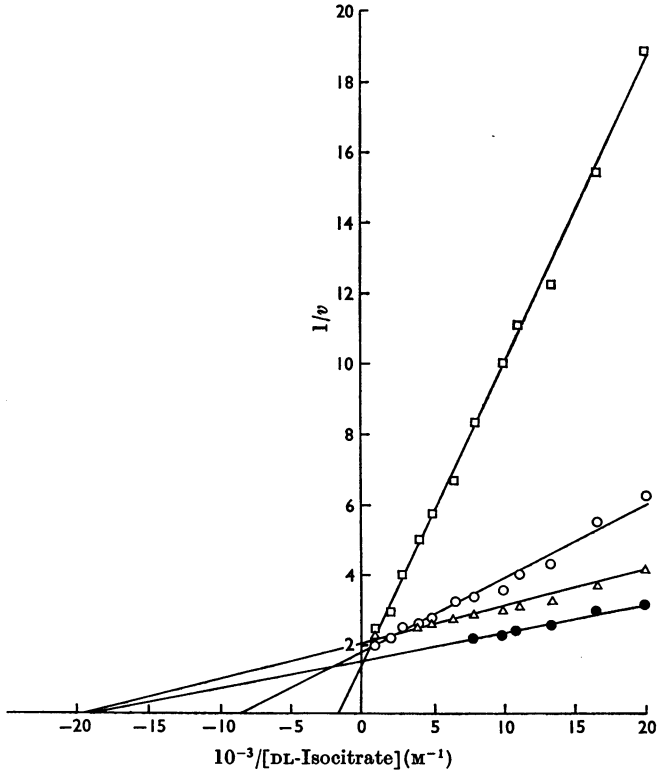

Fig. 9. Effects of α-oxoglutarate on the K_m of DL-isocitrate for the cold-adapted enzyme variant assayed at 10°C. Assays were carried out at pH 8.0 (200mM-tris–HCl, temperature adjusted) with $NADP^+$ and Mg^{2+} saturating. α-Oxoglutarate concentration: ●, none added; △, 0.5mM; ○, 1mM; □, 5mM.

Browning, 1969). This inhibition of NADP–isocitrate dehydrogenase by α-oxoglutarate is of particular interest, since *in vivo*, control of the NADP-linked enzyme could be co-ordinated with adenylate control of trout liver citrate synthase (Hochachka & Lewis, 1970). *In vivo*, under most circumstances, high α-oxoglutarate concentrations would be synonymous with high ATP concentrations. Hence inhibition of the citrate synthase could be achieved by high ATP concentrations in concert with NADP–isocitrate dehydrogenase inhibition by high α-oxoglutarate concentrations.

Trout liver NADP-linked isocitrate dehydrogenase is also inhibited by the concerted action of glyoxylate and oxaloacetate (Table 4). This inhibition has also been observed by Hampton & Hanson (1969) and Ozaki & Shiio (1968) in bacteria, and by Marr & Weber (1969*c*) in protozoa. The significance of this action is not known in higher animals.

Effects of temperature acclimatization on enzyme activities. One general kind of mechanism that has been proposed previously for achieving rate compensation during thermal acclimatization involves changes in the steady-state activities of enzymes. Thus it is known that total 6-phosphogluconate dehydrogenase and aldolase activities (Jankowsky, 1968), total phosphofructokinase activities (Freed, 1969) and total lactate dehydrogenase activities (Hochachka, 1965) appear to be increased during cold acclimatization. Although we have not examined this problem in the case of trout liver NADP–isocitrate dehydrogenase in detail, it is evident from Fig. 5 that total NADP–isocitrate dehydrogenase activities in liver preparations from both cold- and warm-acclimatized trout seem to be about the same. If there is any acclimatization effect it is to slightly lower the activity of the enzyme during cold acclimatization. And indeed, as we have argued above, the two basic forms of this isocitrate dehydrogenase appear to be best adapted for function at their respective thermal ranges. *A priori* it is clear that the production of more of the warm-adapted enzyme variant during cold-acclimatization (or vice versa) would not make sense biologically. As in the case of trout muscle pyruvate kinase (Somero, 1969) and brain acetylcholinesterase (Baldwin & Hochachka, 1970), a more functional solution to the problem of low temperature appears to be to increase the relative steady-state concentrations of an enzyme variant that is better adapted for controlled function at the low temperatures encountered by the organism in Nature.

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