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EFFECTS OF TEMPERATURE ON L-LEUCINE TRANSPORT IN TOADFISH LIVER IN VIVO

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Summary

The effect of body temperature in the 4—30°C range on L-leucine uptake by toadfish liver in vivo was examined by means of a single-injection pulse technique. The ratio of [^{14}C]leucine to [^{3}H]mannitol or [^{3}H]inulin in blood leaving the liver was measured as a function of time after hepatic portal vein injection. Recoveries of the two isotopes in liver and [^{14}C]leucine incorporation into protein were determined.

The Q_{10} value for influx was 3.8, that for efflux 2.8. At all temperatures, the leucine influx was 8—10-times higher than its incorporation into protein. The directly energy-linked reactions appear to be the main site of increased temperature sensitivity at low temperatures.

Introduction

The effects of temperature on membrane function and transport processes have particular relevance for poikilothermic organisms which function over a broad range of body temperatures. The oyster toadfish, a highly adaptable marine teleost of the eastern United States, tolerates temperatures of 0—30°C. At the upper end of its range, it maintains a rate of liver protein synthesis comparable to that in small mammals when temperature difference is taken into account [1]. Amino acid transport systems also are highly active in toadfish liver [2]. Uptake of L-leucine in vivo occurs through a combination of facilitated diffusion and active transport [3]. The present study examines the effect of various temperatures on this system in toadfish liver.

Materials and Methods

Animals. Adult toadfish, *Opsanus tau*, body wt. 310 ± 30 g, liver wt. 6.0 ± 2.1 g, were obtained by local collectors at Woods Hole, MA. Fish were maintained in running sea-water aquaria at ambient summer temperature, 21 ± 1°C, and fed killifish and mussel meat on alternate days. Food was witheld for 48 h prior to experiments. Fish were transferred to heated or cooled aquaria for 1—2 h for adjustment of body temperature to the desired experimental temperature.

Experimental procedure. Anesthesia, hepatic portal vein injection and sample preparation were carried out as described [2,3]. The injection solution contained 2 μCi of L-[U-^{14}C]leucine and 4 μCi of D-[1-^{3}H(n)]mannitol or of [G-^{3}H]inulin (New England Nuclear) in a balanced salt solution at pH 7.4 [4]. The injection was administered slowly enough (5—10 s) to insure mixing with portal blood. Incubation times varied from 1 to 3 min at 21—30°C, 0.5 to 10 min at 10—16°C and 3 to 12 min at 4—7°C. At the end of the incubation period, the liver was quickly excised and blood was collected at the site of the severed hepatic vein. Temperature in the body cavity was measured (±1°C). Free radioactivity for the two isotopes was determined in the acid-soluble fraction of plasma and liver by double-isotope scintillation counting in Biofluor (New England Nuclear). Protein-bound radioactivity in liver was analyzed on filter paper discs [5]. Results were converted to microcuries recovered in the plasma sample and in the entire liver and expressed as fractions of the injected dose [3].

Kinetic analysis. The levels of radioactivity recovered in the various compartments of liver were analyzed according to the following reactions:

$$A_{\text{body}} \underset{k_3}{\overset{k_5}{\rightleftharpoons}} A_e \underset{k_4}{\overset{k_1}{\rightleftharpoons}} A_i^{\text{free}} \overset{k_2}{\rightarrow} A_i^{\text{protein}}$$

$$B_{\text{body}} \underset{k_3}{\overset{k_5}{\rightleftharpoons}} B_e \qquad (1)$$

where the terms from left to right represent the fraction of the injected amino acid (A) in the body excluding the liver, in liver extracellular space, in liver intracellular space as free amino acid, and in liver protein. The rate constants, k_3 and k_5, describe the passage of the injection bolus through the liver and are evaluated from the distribution of the marker (B) between liver (B_e) and the rest of the animal (B_{body}). Inulin and mannitol were used as markers in this study. Inulin occupies only extracellular space in toadfish liver (0.31 ml/g wet wt.); mannitol enters intracellular space slowly [2].

The rate constants, k_1 and k_4, describe influx and efflux, respectively, of the amino acid in liver; k_2 refers to the rate of incorporation into protein. Evaluation of rate constants was based on the experimental measurements of total liver free radioactivity of amino acid ($A_e + A_i^{\text{free}}$), and marker (B_e) and of incorporation into protein (A_i^{protein}). The compartments, A_{body} and B_{body}, were calculated by difference, assuming no isotope was lost from the animal

over these short periods. The fraction of A associated with extracellular space was estimated by use of the equation:

$$A_e = B_e(A/B)_{plasma} \quad (2)$$

where $(A/B)_{plasma}$ is the ratio of the two isotopes, normalized to dose, in plasma obtained from blood draining from the hepatic vein. This ratio is taken to represent the ratio of the isotopes within the extracellular space of liver.

Rate constants which gave the best fit to the experimental data were obtained by successive numerical integration of differential equations based on Eqn. 1. Two time periods were used in the kinetic analysis, as in previous studies [3]. The first, amounting to 22 s at 10°C, represents the period that the entire injection bolus is retained in the liver. For this period, k_3 and k_5 are set to zero; subsequently, values of k_3 and k_5 are determined from the rate of loss of marker from liver.

Results

Effect of temperature on leucine:marker ratios in plasma

The ratio of amino acid to the extracellular space marker in blood leaving the liver immediately after the pulse provides a measure of intracellular uptake of the amino acid by liver. The ratio of the two isotopes in the injection solution was normalized to 1.0. In the case of leucine, the ratio in effluent blood is relatively constant throughout the experimental period of interest. The level of the marker in liver, however, declines during this period from about 60% of the original dose to 10% [3].

Fig. 1 shows the effect of temperature change on the plasma ratio of leucine:mannitol (at 10 and 21°C) or leucine:inulin (at all other temperatures). Experimental times were chosen to obtain similar levels of recovery of the marker in liver (about 20% of dose). At temperatures of 21°C and higher, values for $(A/B)_{plasma}$ are about 0.4, representing a significant level of amino acid extraction by the liver. The plasma ratio increases as temperature is reduced, reaching values as high as 0.8 at 4°C. This indicates a less effective extraction of leucine by liver.

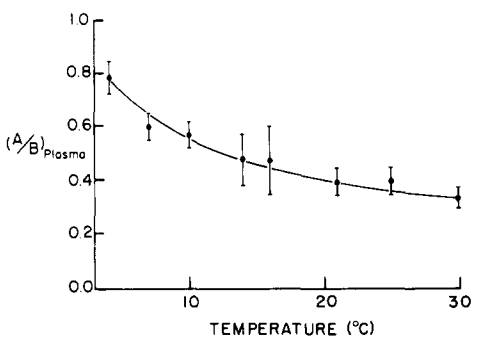

Fig. 1. Plasma ratios of L-[^{14}C]leucine relative to the marker in blood draining from liver following hepatic portal vein injection in vivo. Bars represent S.D. (n = 27 at 10°C, 30 at 21°C, and 3—6 at each of the other temperatures.) The marker is [^{3}H]mannitol at 10 and 21°C and [^{3}H]inulin at all other temperatures. Average B_e was 0.20 ± 0.05.

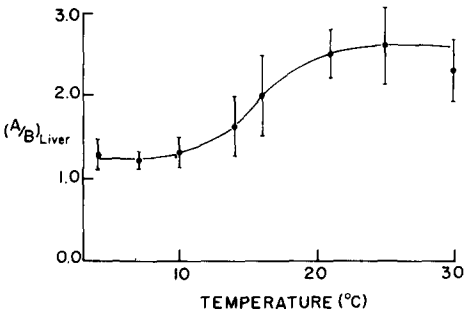

Fig. 2. Ratio of recovery of [^{14}C]leucine in liver relative to the marker following hepatic portal vein injection at various body temperatures. Bars represent S.D. Experimental details are given in Fig. 1.

Effect of temperature on [^{14}C]leucine retention by liver

Fig. 2 presents the temperature dependence of the recovery of radioactive leucine in liver relative to the marker. At all temperatures, the level of amino acid in liver exceeds that of the marker, indicative of amino acid uptake into intracellular space. However, the data fall into two distinct temperature ranges. At 20°C and above, (A/B) ratios in liver average 2.5, based on all recovered amino acid (including protein-bound isotope), or 2.0 based on free radioactivity only. At the lowest temperatures studied (4—7°C), ratios averaged 1.3. Incorporation into protein was negligible at these temperatures.

Kinetic analysis at 10°C

The kinetic behavior of the leucine transport system at low temperatures was examined by carrying out a complete time series analagous to previous studies at 21°C [3]. Movement of the injection fluid through the liver was monitored with [^{3}H]mannitol. Mannitol recovery in liver declined exponentially with time (Fig. 3), yielding the constants, $k_3 = 0.7$ min^{-1} and $k_5 = 0.08$ min^{-1}, for exchange of isotope between liver and body at short times after the pulse. Averaged data for the plasma isotope ratio as a function of B_e are presented in Fig. 4. The ratio is observed to remain nearly constant with time as the marker

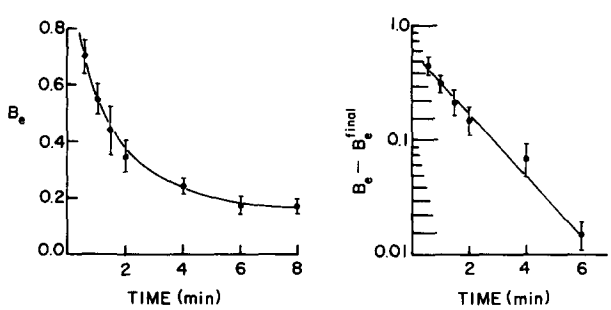

Fig. 3. (a) Recovery of D-[^{3}H]mannitol in toadfish liver as a function of time after hepatic portal vein injection at 10°C. B_e is the fraction of the injected dose retained by liver. The bars represent S.D. (n = 3 at each time). (b) Semilogarithmic plot of the data of a. $B_e^{final} = 0.18$ has been subtracted from B_e. The slope of the line yields $k_3 = 0.7$ min^{-1}; k_5 was calculated from the steady state of B_e at $t = 6$ min.

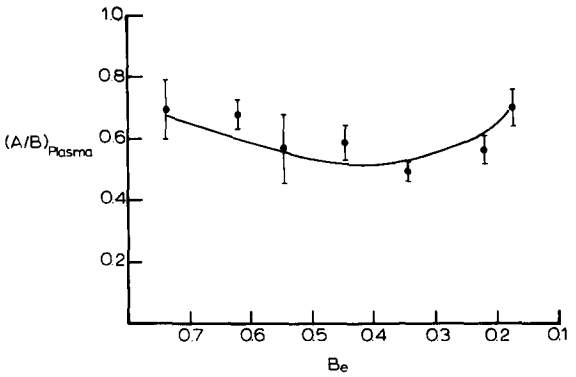

Fig. 4. Plasma ratio of [^{14}C]leucine : [^{3}H]mannitol as a function of mannitol recovery in liver (B_e) obtained at various times after injection. Five to seven animals have been averaged for each point and standard deviations are shown. The curve shown is a theoretical one for A_e/B_e vs. B_e based on the rate constants of Fig. 5.

is lost from the liver. Previous analysis [3] has shown this relation to be strongly dependent on the ratio of influx to efflux rate constants (k_1/k_4). The relation of the two rate constants may be expressed in the form:

$$k_4 = k_1(1.0 - R)/1.5 \tag{3}$$

where R is a parameter varying from 0 to 1. The value 1.5 in the denominator is the ratio of volumes of intracellular to extracellular space in toadfish liver [2]. The case $R = 0$ yields $k_1/k_4 = 1.5$, representing a pure exchange transport process. At the other extreme, $R = 1$ represents one-way transport (influx) only. In the present analysis, the best fit to the experimental data was obtained with $k_1/k_4 = 1.75$ or $R = 0.14$. The theoretical curve in Fig. 4 is based on this value.

Fig. 5 shows the time course for recovery of intracellular free [^{14}C]leucine in liver and the fraction of intracellular uptake incorporated into protein. A_i^{free} was obtained from total free radioactivity in liver after subtraction of the estimated extracellular component according to Eqn. 2. These data were

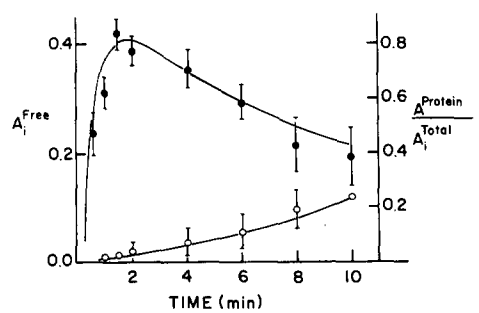

Fig. 5. Recovery of L-[^{14}C]leucine in the intracellular free pool of liver (•, left-hand scale) and the fractional incorporation of intracellular [^{14}C]leucine into protein (○, right-hand scale). Experimental data presented as averages with bars for S.D. (total $n = 52$). Theoretical curves are shown for the following rate constants: $k_1 = 0.70$, $k_2 = 0.02$, $k_3 = 0.70$, $k_4 = 0.40$, $k_5 = 0.08$, all in units of min^{-1}.

used together with those of Fig. 4 to fix the values of k_1 and k_2 in Eqn. 1. Theoretical curves based on the complete set of rate constants are shown in Figs. 4 and 5.

Discussion

Studies of temperature dependence provide a means to evaluate activation energies and mechanisms of carrier-mediated transport processes. Results in a variety of eukaryotic cells have revealed a complex picture of temperature effects on these processes. In most cases, activation energy is elevated at reduced temperatures, and the Arrhenius plot (log velocity vs. inverse absolute temperature) may show a discontinuity or 'break' [6—11]. Other systems show a gradual increase in activation energy as temperature is lowered [4,12]. Various interpretations of these effects have been offered, involving membrane phase transitions [13], asymmetry in carrier reorientation rates [12] and temperature-dependent equilibria between carrier protein conformations [6].

The toadfish liver offers a unique experimental system for study of biological processes in vivo. The present study provides a profile of L-leucine transport over a range of 4 to 30°C. One of the first points of interest is that transport persists at very low temperatures. This is unlike many other eukaryotic cells in which there is virtually no transport below a minimum temperature, about 15°C for alanine in hamster kidney cells [14], carnitine in rat hepatocytes [15] and D-xylose in yeast [8]. The toadfish liver, however, shows a significant change in transport behavior at reduced temperatures as in other systems [4,6—12]. The transition results in a loss of ability to extract the amino acid from the blood (Fig. 1) and a decline in the measured accumulation of leucine in liver (Fig. 2).

Kinetic analysis yields temperature coefficients for various parameters of the system, as modeled in Eqn. 1. Comparison of the mannitol data of Fig. 3 with previous results at 21°C [3] indicates an Arrhenius activation energy of 17 kJ/mol (Q_{10} = 1.3) for k_3 and k_5, both of which reflect circulation rate. Half-times for the loss of mannitol from liver, based on k_3, are 1.0 min at 10°C and 0.8 min at 21°C. These results are consistent with other studies of the temperature dependence of blood flow in fish [16]. Protein synthesis, as described by k_2, shows a very strong temperature dependence as in previous studies [1]. The value of $k_2 = 0.02$ min^{-1} represents a 2% turnover of the intracellular free leucine pool per min.

The most striking change with temperature in this transport system is found in the rate constants k_1 and k_4. Comparison of the best-fitting values at 10°C (Fig. 5) with those obtained at 21°C [3] indicates two different temperature responses. The efflux process (k_4) yields $Q_{10} = 2.8$ ($E_a = 65$ kJ/mol), a value typical of biochemical reactions including carrier-mediated transport. The maximal velocity of α-aminoisobutyric acid transport in rat hepatocytes, for example, shows $E_a = 67$ kJ/mol in the temperature range 22—37°C [17]. In contrast, the parameter for leucine influx (k_1) in toadfish liver shows Q_{10} = 3.8 ($E_a = 85$ kJ/mol).

Comparison of the k_1/k_4 ratios at the two temperatures suggests a possible interpretation. In this system, a value of $k_1/k_4 = 1.5$ is expected for transport

involving facilitated diffusion or exchange processes only [3]. At 21°C, the best-fitting rate constants gave $k_1/k_4 = 2.5$, a value consistent with a concentrative or active transport component amounting to 40% of influx ($R = 0.4$ in Eqn. 3). The data at 10°C, in contrast, yield an estimate of $R = 0.14$ or 14% of influx that is concentrative. It must be noted that the absolute values may be influenced by assumptions in the model. The qualitative change, however, is consistent with the temperature profile for leucine accumulation (Fig. 2).

The gradual change in character of the system from high to low capacity is similar in some respects to the triphasic Arrhenius plot obtained in studies of glucoside transport in bacteria [18]. In that system, activation energies are normal at the highest and lowest temperatures examined; increased activation energy is found only in the transition region (10--20°C) where carriers are presumed to partition between fluid and ordered domains of the membrane. The transition in toadfish occurs in the same temperature range; however, increased temperature sensitivity appears to be limited to influx processes.

In studies of leucine transport by human red blood cells, Hoare [4] has obtained evidence for a loss in net influx at low temperature compared with exchange. Analysis of entry, exit, and exchange kinetics in this system suggested a mechanism involving differential temperature effects on the reorientation rates of loaded and empty carriers. Such a mechanism could account for the present results. Another possibility is that multiple carriers with differing temperature sensitivities are used. Results in Ehrlich ascites cells have suggested loss of carrier specificity at low temperatures [19].

Transport and protein synthesis appear to be closely coordinated in this system. Protein synthesis in toadfish liver shows a normal temperature dependence ($E_a = 67$ kJ/mol) in the 17—30°C range with an increase to 110 kJ/mol below 17°C [1]. At 21°C, leucine influx is about 0.6 μmol/min per 7-g liver, or 10-times the rate of leucine incorporation into protein [3]. Leucine influx at 10°C, based on k_1 and plasma leucine concentration (0.1 mM), is 0.16 μmol/min per 7-g liver. Leucine incorporation into protein at 10°C is 0.021 μmol/min [1]. Hence, the high ratio of transport to synthesis is maintained at 10°C, with both energy-requiring processes showing increased temperature sensitivity at low temperatures.

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