J. exp. Biol. **129**, 191–203 (1987)

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TEMPERATURE AFFECTS THE DIFFUSION OF SMALL MOLECULES THROUGH CYTOSOL OF FISH MUSCLE

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Accepted 20 January 1987

SUMMARY

Undiluted cytosolic extracts were prepared from fast glycolytic muscle tissue of white perch (*Morone americanus*). Diffusion coefficients (D) through the cytosol preparations were estimated *in vitro* for a series of selected low molecular weight compounds using an experimental diffusion chamber. Determinations were made at 5° and 25°C to assess thermal sensitivity of the process. Non-metabolizable analogues of naturally occurring compounds were employed to avoid chemical alteration of solutes by the catalytically competent preparations during diffusion experiments. Kinematic viscosity of cytosolic extracts, which is a major determinant of diffusive resistance, increases from $2{\cdot}94 \pm 0{\cdot}06$ to $5{\cdot}35 \pm 0{\cdot}02 \times 10^{-2}\,cm^2\,s^{-1}$ between temperatures of 25° and 5°C ($Q_{10} = 1{\cdot}35 \pm 0{\cdot}01$). The diffusion coefficients (D) of D-lactic acid are $2{\cdot}26 \pm 0{\cdot}84$ and $0{\cdot}79 \pm 0{\cdot}15 \times 10^{-6}\,cm^2\,s^{-1}$ at 25° and 5°C, respectively ($Q_{10} = 1{\cdot}84 \pm 0{\cdot}36$). The D values of 2-deoxyglucose are $2{\cdot}87 \pm 1{\cdot}01$ and $1{\cdot}22 \pm 0{\cdot}36 \times 10^{-6}\,cm^2\,s^{-1}$ at 25° and 5°C ($Q_{10} = 1{\cdot}75 \pm 0{\cdot}54$). The D values of Ca^{2+} are $2{\cdot}47 \pm 0{\cdot}28$ and $1{\cdot}09 \pm 0{\cdot}36 \times 10^{-6}\,cm^2\,s^{-1}$ at 25° and 5°C ($Q_{10} = 2{\cdot}04 \pm 0{\cdot}36$). The D values for the ATP analogue, AMP-PNP, are $0{\cdot}87 \pm 0{\cdot}33$ and $0{\cdot}81 \pm 0{\cdot}15 \times 10^{-6}\,cm^2\,s^{-1}$ at 25° and 5°C ($Q_{10} = 0{\cdot}98 \pm 0{\cdot}12$). AMP-PNP is the only compound tested which did not show significant thermal sensitivity of diffusion. Recently reported changes in muscle cell ultrastructure induced by temperature acclimation of fishes may serve to counteract the effect of temperature change on diffusion of key small molecules through the aqueous cytoplasm, thus maintaining flux rates between cellular compartments. These mechanisms may be of considerable import in achieving relative temperature independence of cellular function that is characteristic of many eurythermal aquatic animals.

INTRODUCTION

The large changes in body temperature which are experienced seasonally by eurythermal temperate zone fishes present major metabolic challenges requiring physiological compensation if the animals are to maintain relatively constant biological activity. *A priori*, at least three broad areas of thermal sensitivity can be

Key words: temperature, diffusion, cytosol, muscle.

identified: (1) membrane permeability/transport of ions and metabolites; (2) catalytic rate of enzymes in pathways of intermediary metabolism; and (3) diffusive processes of metabolites and respiratory gases within tissues and cells. The first of these, permeability, has received little direct attention from the standpoint of supply of metabolic fuels. The second, catalytic rate, has garnered the lion's share of experimental effort during recent decades and has been reviewed extensively (e.g. see Hazel & Prosser, 1974; Hochachka & Somero, 1984). The last, effects of temperature change on molecular diffusion, has not been considered thoroughly since the early treatments of Krogh (1919).

Molecular diffusion as a potentially limiting factor in physiological processes of other systems, however, has become a topic of increasing interest in recent years (reviewed by Jones, 1986; Masters, 1977; Clegg, 1984). Many studies have further underlined the importance of cytoplasmic viscosity as a major determinant of the rate of intracellular diffusion of small compounds (Mastro, Babich, Taylor & Keith, 1984; Gershon, Porter & Trus, 1985; Wojcieszyn, Schlegel, Wu & Jacobson, 1981). The importance of these factors to animals which experience significant changes in body temperature becomes apparent in the light of the thermal sensitivity of viscosity of aqueous solutions. For example, even pure water undergoes a 71 % increase in viscosity (from 0·89 to $1{\cdot}52\times10^3$ Pa·s) between 25° and 5°C (Weast, 1971), a range which encompasses the natural thermal regime of many temperate-zone fishes.

In recent years, several laboratories have reported that major ultrastructural changes in muscle tissue of fishes are induced by thermal acclimation (Johnston & Maitland, 1980; Penney & Goldspink, 1980; Tyler & Sidell, 1985; Egginton & Sidell, 1986). These results have prompted the suggestion that cold-induced proliferation of organelle compartments in fish muscle may serve to ameliorate the effect of temperature upon diffusion of small compounds between cellular compartments (Sidell, 1983). To evaluate this hypothesis we needed to quantify the magnitude of temperature effects upon cellular diffusivity constants of small molecules.

While recognizing the caveats raised by Clegg (1984) in his excellent review on properties of the aqueous cytoplasm, several lines of experimental evidence suggested that the problem for muscle tissue might be addressed using an *in vitro* approach. Caille & Hinke (1974) have estimated that 80 % of cellular water in muscle fibres is accessible to diffusion of small molecules. They and other workers have also shown that diffusion of small molecules in muscle tissue conforms to the expectations of the one-dimensional diffusion equation (Kushmerick & Podolsky, 1979; Bunch & Kallsen, 1969). Finally, the environment through which small molecules diffuse in intact cells appears to have physical characteristics consistent with an aqueous solution (Mastro *et al.* 1984).

The objectives of the study reported here were: (1) to quantify the impact of temperature upon cytosolic viscosity and diffusion coefficients of small molecules moving through cytosolic fluid; and (2) to correlate the magnitude of these effects with predictions based upon changes in muscle ultrastructure which are induced by thermal acclimation. To approach these objectives, we obtained undiluted cytosolic

preparations from white muscle tissue of white perch (*Morone americanus*). Although structurally disrupted, these preparations should still contain all the constituents of the tissue's aqueous cytoplasm. Viscosities of the preparation were determined at warm and cold temperatures and diffusivity constants of selected small molecules in the cytosol were estimated at 5° and 25°C using critically machined diffusion chambers.

Our results suggest that changing body temperature may have significant impact upon diffusive flux of low molecular weight compounds through muscle cells of fish. This limitation may be a causative factor in inducing ultrastructural changes of the tissue in response to long-term changes in body temperature.

MATERIALS AND METHODS

Animals

White perch (*Morone americanus*) were captured by trap net from Mud Pond, Old Town, Maine. After transport to the University of Maine, fish were held in 1000-l circular tanks in filtered, aerated well water at 25°C (± 0·1°) for a minimum of 6 weeks prior to experiments. Animals were fed daily with a controlled diet (Stone & Sidell, 1981) and photoperiod was maintained at 12h:12h (L:D) with gradual excursion (40 min) between full light and darkness controlled by a sunrise–sunset simulator (Conviron, Inc.).

Preparation of muscle cytosol

White muscle tissue was used as a source for cytosolic preparations because it represents a large percentage of body mass and it has an extremely small extracellular space and low capillarity. The latter features minimize contamination by extracellular non-cytosolic fluid.

Animals were removed from tanks and killed by a stunning blow to the head followed by decapitation. Residual blood in the animal's trunk musculature was minimized by massaging the trunk to strip partially the muscle vasculature of fluid. White muscle was dissected carefully from the epaxial mass, taking care to avoid contamination by the small amount of red muscle tissue concentrated around the lateral line region. All dissection procedures were carried out in ice-chilled trays or containers. Once removed, white muscle tissue was weighed, then minced with scissors into small pieces. Minced tissue was transferred without any additional liquid to a small ice-chilled container and homogenized to a uniform paste-like consistency with a Virtis '23' homogenizer. The resulting muscle paste was packed into centrifuge tubes and spun for 30 min at approximately 40 000 ***g*** in a refrigerated centrifuge. Supernatant fluid (crude cytosol) was removed carefully by Pasteur pipette and then cleared of any residual particulate matter by passing through a 0·2 μm syringe filter (Schleicher & Schuell, grade BA83). The volume of the resulting cytosolic fluid was recorded. To eliminate the possibility of bacterial growth during the extended time necessary to perform diffusion experiments (see

below), crystalline NaN_3 was dissolved in the cytosolic fluid to a concentration of 0·2% (w/v) after the viscosity had first been determined.

Viscosity determinations

Kinematic viscosity of each cytosolic preparation was determined at both 5° and 25 °C after thermal equilibration of both fluid and apparatus. The mean of three determinations with Canon–Fenske viscometers (EXAX sizes 50 or 100) was recorded. Because the density of aqueous solutions changes less than 1% between 25° and 5 °C, we chose to determine kinematic viscosity of the fluid. Kinematic viscosity could be estimated by experiment more easily than absolute viscosity (in Pa · s). This same feature should ensure that our estimates of kinematic viscosity accurately reflect equivalent thermal alterations in the absolute viscosity of cytosolic preparations.

Diffusion experiments

The experimental chambers and methods of data analysis employed in our diffusion experiments are described in detail elsewhere (J. R. Hazel & B. D. Sidell, in preparation), but will be briefly summarized below.

Apparatus

The device used for determination of cytosolic diffusivity constants of metabolites was machined from cast acrylic rod and consisted of three compartments arranged in series (Fig. 1). The two end compartments accommodated small (9 mm) magnetic stirring bars and were sealed at the top with adjustable acrylic pistons fitted with Neoprene O-rings. Pistons could be vented or closed by means of a removable threaded nylon fastener which permitted access for sampling but maintained constant the volume of each compartment during the experiment. Each end compartment was separated from the critically machined central compartment (through which diffusion took place) by 0·2 μm membrane filters (Schleicher & Schuell, type BA83, lot no. 4129/2) which prevented bulk transfer between compartments and were held in position by pliable gaskets formed with clear Sylgard 184 elastomer (Dow Corning, Midland, MI). Because the effective surface area and pore thickness of nitrocellulose membranes were not known precisely, cell constants (K) were determined by calibrating the apparatus by experiments conducted with water as solvent and a solute of known diffusion coefficient in water, namely KCl (D at 25 °C in water = $1{\cdot}83\times10^{-5}\,cm^2\,s^{-1}$; Harned, 1947).

Protocol

At the initiation of the diffusion run, cytosol was subdivided into two samples. Into the smaller of these was dissolved sufficient crystalline solute under study to achieve the desired starting concentration for one end compartment. This solution (approximately 3·5 ml) was used to fill the end compartment, designated as the supply or injection side. Equivalent cytosol but without added solute was used to fill

both the central and opposing end compartments, the latter designated as the sample or receiving side. Initial samples were removed for determination of solute in both injection (C_i) and receiving (C_r) compartments, thus defining the initial concentration gradient (ΔC_0). Paired chambers containing identical cytosol preparations were equilibrated at each temperature (5°, 25°C). Chambers were maintained at 5° (in a cold room) or at 25°C (by partial submersion in a water bath) throughout each experiment.

Each end compartment was mixed continuously by low-speed magnetic stirring. Control experiments established that stirring did not result in convective bulk transport through the membrane filter. At varying time intervals (duration dependent upon temperature and solute), 50-μl samples were removed from both injection and receiving sides for subsequent analysis of solute concentration. These data permitted both description of the instantaneous concentration gradient between end compartments at each sampling time and calculation of mass of solute transferred during the time interval since the previous sample.

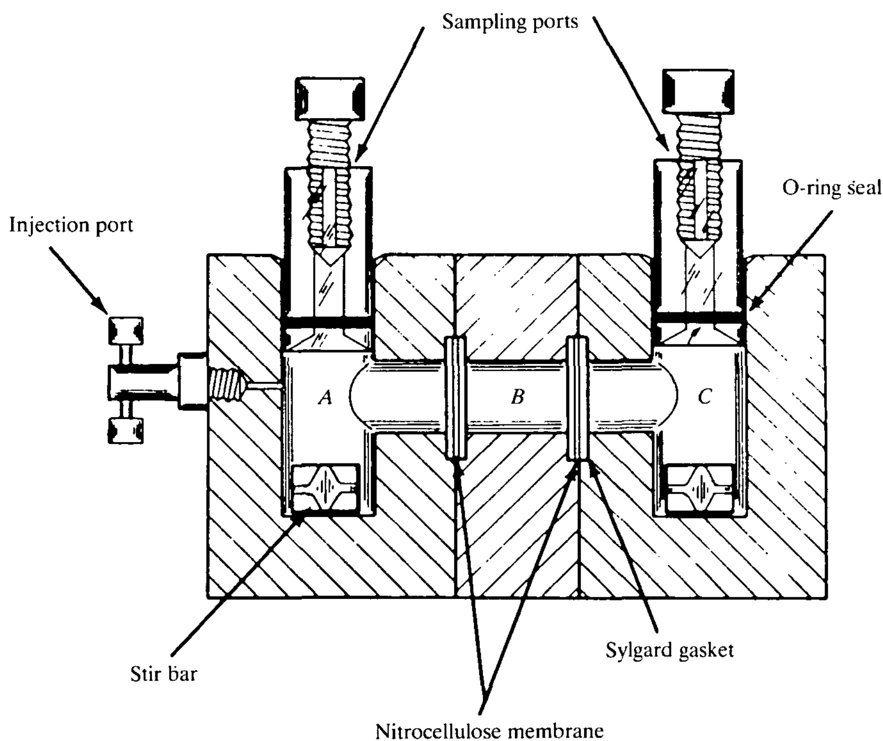

Fig. 1. The three-chambered constant-volume apparatus used for determination of diffusion coefficients through muscle cytosol. The central compartment was separated from the end compartments by a 0·2 μm membrane filter which allowed free movement of solutes but prevented convective exchange of fluid (see text for detailed description). The mininert valve for injection of solute is shown in this diagram at the end of the chamber; location of the valve in the actual apparatus was at the front surface of the chamber, 90° from the location depicted. *A*, injection side; *B*, central compartment; *C*, receiving side.

Analyses of solute concentrations

Because our cytosolic preparations were capable of catalysing reactions involving many intracellular metabolites, it was necessary to utilize non-metabolizable analogues of biologically important compounds. On this criterion we chose to estimate diffusivity constants of the following compounds.

(1) D-Lactic acid. This isomer of lactic acid cannot serve as a substrate for the L-lactic-acid-specific lactate dehydrogenase (LDH) of vertebrate tissues. The concentration of D-lactate in neutralized 6% $HClO_4$ (PCA) extracts of our samples was determined enzymatically utilizing bacterial D-lactic-acid-specific LDH from *Lactobacillus leichmanii*.

(2) 2-Deoxyglucose (2-DOG). This non-metabolizable analogue of glucose was used as a representative hexose. Concentrations in neutralized PCA extracts of samples were determined chemically by assay for total reducing sugars utilizing the anthrone procedure described by Umbreit, Burris & Stauffer (1972), subtracting out background colour development at 620 nm determined for cytosol containing no added sugar. Standard curves were constructed from solutions containing known amounts of 2-DOG.

(3) 5′-Adenylyl-imidodiphosphate (AMP-PNP). This compound is a structural analogue of ATP in which the oxygen of the normally labile terminal ester linkage is replaced by a nitrogen in an imido linkage. The terminal phosphate is thus not subject to hydrolysis by the normal action of cytosolic ATPases. The concentration of this compound was assayed radiochemically by liquid scintillation counting. Labelled 5′-adenylyl-2,8-[^{3}H]imidodiphosphate (tetrasodium salt, ICN, Irvine, CA) was added to a larger mass of unlabelled AMP-PNP carrier at the initiation of the experiment.

(4) Calcium. The absolute free calcium concentration of cytosol was determined by organometallic dye-binding using murexide (Scarpa, Brinley, Tiffert & Dubyak, 1978). Diffusion of the compound was followed radiochemically by liquid scintillation counting. $^{45}Ca^{2+}$ (New England Nuclear) was added to a larger mass of unlabelled $CaCl_2$ carrier at the initiation of the experiment.

All liquid scintillation (LSC) procedures were carried out using a Packard Tri-Carb 3255 counter and the xylene-based LSC cocktail of Anderson & McClure (1973). All chemicals were of reagent grade or higher purity and, unless otherwise noted, obtained from Sigma Chemical Co. (St Louis).

Data analysis

Our measurements allowed us to define the instantaneous concentration gradient (ΔC_i) for the solute of interest at each sampling time. Measurements of the incremental increase in concentration of the receiving chamber also permitted description of the accumulative mass flux (J_{acc}) from the injection side to the sample side during the course of the experiment.

The one-dimensional diffusion equation is:

$$J_i = dN/dt = -D \times K \times (\Delta C_i),$$

where J_i is instantaneous flux (in μmol s^{-1}), ΔC_i is instantaneous concentration gradient (in μmol cm^{-3}), D is diffusion coefficient of substance N (in $cm^2 s^{-1}$), and K is a constant (the cell constant) representing the effective surface area across which diffusion is occurring divided by the effective length of the diffusion path ($cm^2 cm^{-1} = cm$).

To solve the equation for D, it was necessary to obtain a comparable instantaneous estimate of flux (J_i) to that measured for concentration gradient (ΔC_i) at each sampling time. During the course of the experiment, the driving diffusional concentration gradient (ΔC_i) decays in a regular exponential fashion resulting in equivalent exponential decay of accumulative mass flux (J_{acc}; see example data, Fig. 2). Consequently, it was possible to obtain estimates of J_i at each sampling time by determining the first derivative of the curve-fitting equation describing J_{acc} *versus* time. Curve fitting was accomplished using the iterative KINFIT program of Knack & Rohm (1981).

A plot of J_i (derived as described above) *versus* ΔC_i (measured) thus yields a straight line with slope equal to $D \times K$ (see example data in Fig. 3). Estimates of D for the compound under study were obtained easily from linear regression analysis of this plot. Estimates of D for each compound and for cytosolic viscosity at different experimental temperatures were compared using Student's *t*-tests.

RESULTS

Effect of temperature on cytosolic viscosity

Kinematic viscosities of cytosolic preparations were significantly different ($P < 0.001$) at the two experimental temperatures employed (Table 1). Decreasing

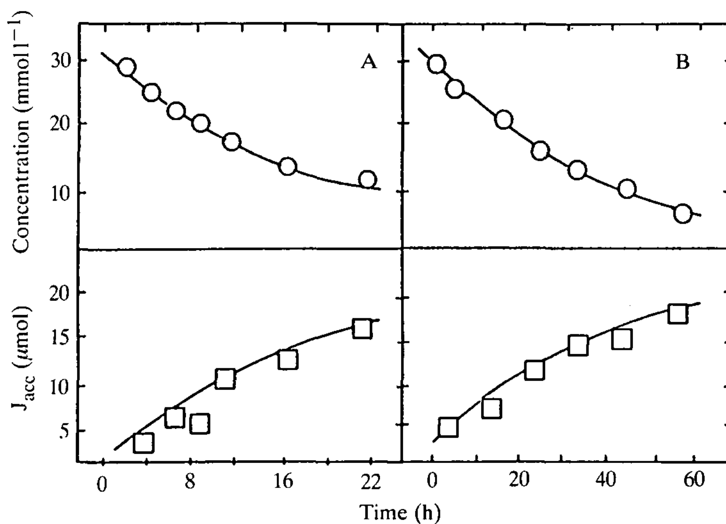

Fig. 2. An example of measurements obtained from diffusion experiment of D-lactate through muscle cytosol in paired chambers at (A) 25°C and (B) 5°C.

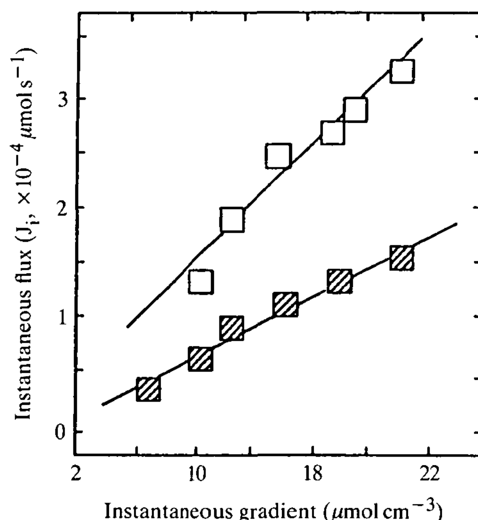

Fig. 3. Plot of derived instantaneous flux (J_i) of D-lactate *versus* measured instantaneous concentration gradient (ΔC_i) for data shown in Fig. 2. Open boxes are for 25°C data while hatched boxes are for 5°C data. Diffusion coefficients were calculated from regression analysis of slopes (see text for detailed explanation of methods).

temperature from 25° to 5°C led to an approximately 1·8-fold increase in cytosolic viscosity.

Determination of diffusivity constants

The experimental protocol and method of data analysis used in our experiments enable us to ascribe confidence limits to estimates of D values for small compounds. Plots of J_i *versus* ΔC_i used to estimate D values (see Fig. 2) appear to reinforce further the validity of our method, since linear regression analyses of these data typically yielded correlation coefficients in the range 0·95–0·98.

At the experimental temperature of 25°C, estimates of D for the solutes tested rank as might be expected given the effects of both molecular size and charge characteristics upon retarding diffusion through the viscous and polyelectrolytic cytosol preparations (Table 2). Diffusivity constant for the polar but uncharged hexose, 2-DOG (relative molecular mass, $M_r = 164{\cdot}16$), is approximately 3·3-fold greater than that of the bulkier ($M_r = 502{\cdot}38$) and highly-charged AMP-PNP. Ratios

Table 1. *Temperature dependence of viscosity of cytosol preparations from white muscle tissue*

Temperature (°C)	Kinematic viscosity ($\times 10^{-2}$ cm^2 s^{-1})	Q_{10}
5	5·35 ± 0·02	1·35 ± 0·01
25	2·94 ± 0·06	

Data shown are mean ± S.E.M.; $N = 12$ for all entries.
Kinematic viscosities were significantly different between 5° and 25°C ($P < 0{\cdot}001$).

Table 2. *Effect of temperature upon diffusion coefficients of selected small molecules in muscle cytosol*

Compound	ΔC (initial) ($mmol\,l^{-1}$)	Diffusion coefficient* ($\times 10^{-6} cm^2 s^{-1}$) 5 °C	25 °C	Q_{10}†
D-Lactate	30	0·79 ± 0·15 (4)	2·26 ± 0·84 (3)	1·84 ± 0·36 (3)
2-Deoxyglucose	75	1·22 ± 0·36 (4)	2·87 ± 1·01 (4)	1·75 ± 0·54 (4)
$^{45}Ca^{2+}$	10	1·09 ± 0·36 (4)	2·47 ± 0·28 (2)	2·04 ± 0·36 (2)
AMP-PNP	5	0·81 ± 0·15 (4)	0·87 ± 0·38 (4)	0·98 ± 0·12 (4)

Data shown are means ± S.E.M. for number of determinations given in parentheses. Estimates of D for each metabolite other than AMP-PNP are significantly different ($P < 0.001$) between experimental temperatures.

* Preliminary communication of these results (Sidell & Hazel, 1984) did not incorporate the use of cell constants ($K = 10.66$; see text), resulting in proportionate overestimates of true diffusion coefficients.

† Q_{10} values were determined from paired chambers containing identical cytosolic preparations and may not reflect exactly those calculated from mean values for D at each temperature.

of diffusivity constants determined in cytosol (D) and in pure water (D_0) at 25 °C using similar methods (J. R. Hazel & B. D. Sidell, in preparation) range from 0·2 for AMP-PNP to 0·4 for lactic acid, indicating that diffusion of small molecules through cytosol occurs between 2 and 5 times more slowly than in dilute aqueous solution.

Decreasing the temperature of the cytosolic preparations from 25° to 5 °C results in a 2·2- to 2·8-fold decrease (Q_{10} approximately 2) in the rates of diffusion of D-lactate, 2-DOG and Ca^{2+} (Table 2). However, estimates of D at 25° and 5 °C for AMP-PNP are not significantly different. Because of this lack of thermal sensitivity of D for the ATP analogue, at the colder temperature it is no longer rank-ordered as the most slowly diffusing substance.

DISCUSSION

Although the issues have been addressed for many decades (e.g. see Krogh, 1919), a lively debate still continues regarding the nature of diffusive processes within the cytoplasm of living cells. Recent reviews have argued that because of the elaborate cytoskeletal and microtrabecular lattice of cells, a large proportion of intracellular water is either bound to structures or in their close vicinity and '...exhibits properties that markedly differ from pure water...' (Clegg, 1984). According to Clegg's view, macromolecular constituents such as many enzymes and proteins, normally associated with the cytosol obtained by cell fractionation procedures, exist *in vivo* in the zone of 'structured' water, while the remaining small proportion of aqueous cytoplasm more closely approximates a dilute solution of metabolites and ions. These arguments are supported by the recent experiments of Mastro *et al.* (1984) who have shown that apparent cytoplasmic viscosities, calculated on the basis of rotation of spin-labelled macromolecules and small molecules in intact cells, differ by orders of magnitude. Spin-labelled proteins yield estimates of cytoplasmic

viscosity as high as $100\text{–}200\times10^{-3}$ Pa · s while small molecules appear to exist in a cellular environment of $2\text{–}6\times10^{-3}$ Pa · s.

Because the issues we address in the present paper deal with the diffusive movement of small molecules, we note with some satisfaction that viscosity estimates of our cytosolic preparations from fish muscle are quite consistent with the description by Mastro *et al.* (1984) for the milieu of small molecular species in intact cells. Indeed, muscle tissue may be ideally suited for simulating cytoplasmic characteristics with *in vitro* preparations since the preponderance of the tissue's structural framework is the myofibrillar lattice of contractile proteins which are pelleted in high-speed centrifugal separations. This conclusion is supported by Caille & Hinke's (1974) observation that approximately 80 % of the water volume (65 % of fibre volume) of barnacle muscle is available for free diffusion of small substances.

The continuing controversy about the compartmental proportions of cellular water has not diminished the consensus that diffusion of oxygen and small molecules is important under cellular conditions and, in some cases, may limit physiological processes (reviewed by Jones, 1986). The results of many studies have also established that the viscosity of cellular fluid is a major determinant of the rate of intracellular diffusion of small molecules (Gershon *et al.* 1985; Wojcieszyn *et al.* 1981). Each of these factors becomes particularly relevant when considering the metabolic challenges to animals whose body temperature may vary seasonally by more than 20°C.

Clearly changes in temperature similar to those experienced naturally by eurythermal fishes at high-temperate latitudes can lead to significant alterations in viscosity of their cellular fluids. Our estimates of thermal dependence of cytosolic viscosity show a greater than 80 % increase as the temperature of the muscular fluid is cooled from 25° to 5°C. These estimates, although obtained *in vitro* with cytosolic preparations, are within the range of values estimated for small molecules within intact cells by non-invasive methods. Solely on the basis of observed changes in viscosity, we would predict, therefore, that diffusive flux of critical metabolites and regulatory ions would be greatly slowed in fishes at winter body temperatures of 0–4°C compared to the 20–25°C range typically experienced during summer months.

If viscosity change were the only factor affecting diffusion of small molecules, we would anticipate that thermal sensitivities observed for movement of the solutes studied would closely match that measured for viscosity over the temperature range of our experiments (i.e. Q_{10} of approximately 1·3). For the solutes measured which do show significant thermal sensitivity of diffusion – lactate, 2-DOG and Ca^{2+} – this factor can account for a large portion but not the entire temperature effect on their diffusion coefficients (see Table 2). Q_{10} values measured for each of these solutes range between approximately 1·75 and 2·0 and are not statistically different. Two factors in addition to viscosity may contribute to the observed depression of D values at colder temperatures: (1) reduced kinetic energy of the system (although a reduction of only <7 % on the absolute temperature scale); and (2) the increase in bond strength of ionic and dipole interactions which accompanies decreasing temperature. The latter may be a significant consideration for movement of charged

molecules through the polyelectrolytic medium of our cytosolic preparations. This may account for the result that the estimate of Q_{10} (D) for the polar but uncharged 2-DOG is not significantly different from that for viscosity while thermal sensitivities for D of ionized lactic acid and Ca^{2+} are greater ($P < 0{\cdot}05$).

In the light of both viscosity effects and charge characteristics mentioned above, perhaps the most surprising result of our study is that estimates of D for the ATP analogue, AMP-PNP, show apparently complete independence of temperature. This unexpected finding may be of considerable significance because of the critical requirement for unimpeded exchange of adenylates between metabolic compartments in order to preserve adenylate acceptor control of mitochondrial respiration (see Tzagoloff, 1982). The mechanistic explanation of the observation, however, is elusive. One possibility is related to the now well-established effect of temperature upon the pH of physiological fluids (Rahn, Reeves & Howell, 1975; Somero, 1981). pH values of our cytosolic preparations averaged approximately 6·7 at 25 °C and were unaltered throughout the duration of experiments. Reduction in temperature to 5 °C would thus result in a pH increase of approximately 0·3 units to a value slightly greater than 7·0. Estimates of pK_5 for ATP at 25°C are in the range of 6·5–7·0 (Sober, 1968) and the pK values of ionizable phosphate groups are little affected by temperature over a wide range (Perrin & Dempsey, 1974). A reversible protonation of one ionizable group on AMP-PNP would thus occur between experimental temperatures of 25° and 5 °C, perhaps altering interaction with the polyelectrolytic cytosol. Ionization of both lactic acid and Ca^{2+}, however, should be unaffected by pH changes in this range.

Although unimpeded movement of adenylates between cytosolic sites of ATP demand and mitochondria is necessary for the maintenance of metabolic regulation, adenosine nucleoside phosphates are not the only commodities which must be exchanged between these two compartments. Many other metabolites and ions move through the cytosol to the mitochondrial interface before being imported by specific transport systems (Tzagoloff, 1982). In addition, Ca^{2+} released from the sarcoplasmic reticulum (SR) of muscle tissue must be able to diffuse rapidly to binding sites on troponin C to activate contraction. The rate of muscular relaxation is also at least partially dependent upon diffusion of Ca^{2+} released from troponin back to SR pump sites for resequestration. Our data suggest that diffusion of many molecular species may be reduced by decreases in cell temperature. Unless exposure to cold induces mechanisms to overcome these depressed rates of diffusive flux, both metabolic and contractile performance of the tissue will be affected adversely. The relatively homeokinetic behaviour of many eurythermal fish species suggests the existence of such adaptive responses. At least two mechanisms can be hypothesized.

All of our data were obtained from *in vitro* manipulation of preparations derived from tissues of 25 °C-acclimated fish. It is at least theoretically possible that cold acclimation could result in decreased viscosity of muscle cytoplasm by alterations in hydration state of the tissue. At present, insufficient data are available to resolve this question directly. However, ultrastructural studies of muscle from thermally acclimated fishes show very little change in the percentage of cell volume occupied by

sarcoplasm, suggesting that major changes in tissue hydration are unlikely (Tyler & Sidell, 1985; Egginton & Sidell, 1986).

A more attractive candidate for overcoming thermal impacts upon diffusion processes is the major quantitative change observed in populations of muscle organelles during the process of temperature adaptation in fish. Acclimation to cold induces marked increases in populations of muscle mitochondria (goldfish: Johnston & Maitland, 1980; Tyler & Sidell, 1985; striped bass: Egginton & Sidell, 1986) and in the volume of sarcoplasmic reticulum (Penney & Goldspink, 1980). Both responses will decrease the mean cytoplasmic diffusion path length for small molecules and increase the surface area of membrane interface between cytoplasmic and organelle compartments. The vector of change in each of these terms will contribute towards offsetting cold-induced decreases in D for solutes traversing the cytoplasm to the organelles. Quantitative estimates of diffusion path lengths and area terms for the cytoplasmic–mitochondrial system have been obtained from stereological analyses of electron micrographs of tissue from goldfish acclimated to 5° and 25°C (Tyler & Sidell, 1985). When these values are substituted into the one-dimensional diffusion equation, it predicts that ultrastructural responses during adaptation from 25° to 5°C could compensate for a 3·1- to 3·4-fold decrease (i.e. $Q_{10} = 1{\cdot}8$) in diffusivity constants of small molecules. These estimates are very similar to the actual thermal sensitivities of cytosolic diffusion constants of small molecules reported in the present study.

In conclusion, we have found that diffusion of small molecules through fish muscle cytosol occurs 2–5 times more slowly than in dilute aqueous solution. Temperature does affect diffusivity for the majority of small molecules. Much, but not all, of the temperature effect observed can be ascribed to changes in cytosolic viscosity. The ultrastructural reorganization observed in fish muscle after temperature acclimation appears to have the potential for counteracting temperature-dependence of diffusion and ensuring maintenance of flux rates between cellular compartments. Presumably these mechanisms are of considerable importance in achieving the relative temperature-independence of cellular function characteristic of many aquatic animals.

The research reported here was supported by US National Science Foundation grants PCM 81-04112 and DCB 85-18442 to BDS and PCM 83-01757 and DCB 86-01195 to JRH. We are particularly indebted to Mr Bernard Goodwin for his skilled work in machining the diffusion chambers.

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