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9. *Heterosporium iridis* (from R. D. Raabe), on irises, primarily *Iris germanica*, produces brown-bordered spots with dark green outer rings; these annular GI's persist while the surrounding tissue yellows. *Septoria lycopersici* (Raabe) causes similar GI's on the tomato plant, *Lycopersicon esculentum*. *Helminthosporium* sp. (strain 1150 of C. I. Kado) produces brown spots, not GI's, on leaves of oats, *Avena sativa*. *Septoria* sp. (Kado strain 1326) causes reddish necrotic spots on *Phlox drummondii*. Small malt agar blocks of all four fungi gave bacterial growth zones several centimeters in diameter on a lawn of *Arthrobacter* Jg-9 (ATCC 25091) in terregens assay medium–agar [A. D. Antoine, N. E. Morrison, J. H. Hanks, *J. Bacteriol.* **88**, 1672 (1964)]; this assay is sensitive to $\geqq 10^{-10}M$ to $10^{-9}M$ siderochromes.
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Solute Concentration Gradients in Frog Muscles at 0° C: Active Transport or Adsorption?

Abstract. *In isolated frog muscle that has been incubated for 7 days at 0°C in vitro K+ and Na+ remain at normal concentrations. Amino acids are accumulated against a concentration gradient at this temperature; for example, glycine accumulates in muscle to a concentration ten times that in the external solution. The amount of cycloleucine accumulated is greater at 0°C than at 25°C. These findings, which are difficult to explain on the basis of metabolically linked active transport, are consistent with the view that solute accumulation by cells is the result of adsorption on specific sites.*

The loss of K^+ and gain of Na^+ associated with cooling of many mammalian tissues are usually considered to be due to a decrease in the rate of metabolic reactions linked to active transport mechanisms (*1*). However, the evidence of Reisin *et al.* suggests that this observation may be explained by a reversible change in Na^+ and K^+ preference of the cell, a change that occurs at a critical temperature, which is below the normal body temperature of the animal (*2*). This change should not occur in tissues from species adapted to temperatures near freezing, so that normal concentration gradients of ions and other solutes would be maintained at 0°C. In support of this concept, I have found that muscle tissue from a northern poikilotherm, Wisconsin *Rana pipiens*, maintains its normal K^+ and Na^+ content and is also able to accumulate amino acids against a concentration gradient during a prolonged incubation at 0°C in vitro.

Four small leg muscles, the sartorius, iliofibularis, semitendinosus, and tibialis anticus longus, were dissected, with all fibers intact, from healthy frogs. After being washed for 6 hours in glucose-free Ringer phosphate (RP) (*3*) at 25°C to reduce the concentration of endogenous amino acids and hormones, the muscles were placed in RP in a watertight vial and immersed in a water bath with continuous slow shaking at either 0° ± 0.1°C or 25° ± 0.5°C. For studies of amino acid accumulation the appropriate concentration of ^{14}C-labeled glycine or cycloleucine, with carrier, was added to the incubation medium. After the desired length of time the tendinous ends were removed from the muscles. The muscles were then blotted, weighed, dried overnight (100°C), and weighed again; the difference in weights was taken as the water content of the sample. The K^+ and Na^+ contents were determined by flame photometry of hot 1*N* HCl extracts of the muscles. Labeled free amino acids were extracted with 5 percent trichloroacetic acid. Aliquots of the extracts were counted with Bray's solution in a liquid scintillation counter.

Figure 1A shows the Na^+, K^+, and water content of frog muscles maintained for periods of up to 12 days at 0°C. The water content remained constant at an average value of 80.6 percent. An initial rise in Na^+ and K^+ content was observed; this increase also occurs after the tissue is incubated for 6 hours at 25°C, and is probably the result of transfer of the muscle from plasma to Ringer solution. The concentrations of both Na^+ and K^+ remained constant from day 1 to day 7, after which K^+ began to fall slowly and Na^+ to rise.

These findings are not due to impermeability of the muscle membrane at this well-controlled low temperature because:

1) In potassium-free solution at 0°C, muscles show a net gain of Na^+ and a net loss of K^+ (Fig. 1B), an indication that both ions are free to cross the cell membrane.

2) The slowly exchanging fraction of labeled Na^+ at 0°C has a half-time of 9.5 hours (*4*).

3) Labeled glucose enters frog muscle at 0°C, reaching a steady-state concentration after 15 hours (*5*).

4) Muscles loaded with Na^+ can accumulate K^+ at 0°C (*6*).

5) Amino acids are accumulated against a concentration gradient by frog muscle at 0°C.

The results in Fig. 2 show that glycine and cycloleucine attain concentrations ten and three times that of the external medium, respectively, at 0°C. This process of accumulation is slow, requiring about 3 days to reach a steady-state concentration. Similar gradients have been obtained with lysine and tryptophan. Although the temperature dependence of amino acid accumulation has been described for a number of tissues (*7*), few studies have been carried out at temperatures as low as 0°C. Cohen and Rickenberg (*8*) reported that the steady-state concentration of valine accumulated by *Escherichia coli* was the same at 0°C as at 37°C, although the process required 120 minutes to come to equilibrium at the lower temperature compared to 1 minute at 37°C. On the other hand, Blasberg and Lajtha (*9*) found much lower concentrations of amino acids in slices of the mouse brain at 0°C than at 37°C. One cannot, however, be certain from their data that a steady state had been attained.

Because cycloleucine is not metabolized by frog muscle at any temperature (*4*), it is possible to compare its accumulation at different temperatures; its final cellular concentration is higher at 0°C than at 25°C (Fig. 2). A similar finding at somewhat higher temperatures has been reported (*10*) for the entry of α-methylglucoside into slices of the kidney cortex and was interpreted by them as representing ". . . a relatively greater inhibitory effect of temperature reduction on the sugar exit process than on the entry mechanism" (*10*). Whatever the mechanism of solute accumulation, the steady-state (or equilibrium) concentration represents the point at which entry and efflux are equal. An agent that brings about an increase in the steady-state concentration of a substance must necessarily decrease its rate of efflux relative to its rate of entry. This, however, does not elucidate the basic mechanisms involved.

It has been accepted that the major portion of intracellular amino acid is free in solution; concentration gradients between the cytoplasm and the external solution are achieved by an active transport process or pump that propels the amino acid into the cell. Efflux from the cell may proceed by a simple diffusive leak or by an exchange mechanism. Before a detailed examination of possible effects of temperature on such mechanisms is carried out, the role of metabolic energy in the production or maintenance of amino acid concentration gradients, as well as ionic gradients, at 0°C, should be determined.

At 0°C, glucose metabolism in frog muscle is reduced to a level which cannot be detected during 16 hours of incubation in vitro (*11*). After 8 days of incubation with labeled glycine, at 0°C, less than 1 percent of the glycine present in the muscle has been metabolized; another 2 to 3 percent has been converted to serine (*4*). After 8 hours of incubation at 0°C, in the presence of iodoacetic acid and in a pure nitrogen atmosphere, the concentrations of Na^+ and K^+ in isolated frog muscle are unchanged (*3*).

These findings suggest that metabolically linked active transport is not the basis for the maintenance of solute gradients in frog muscle at 0°C, and that consideration should be given to the alternative—that the concentrations of Na^+ and K^+ in cells reflect the physical-chemical state of a protein-ion-water matrix making up both cytoplasm and cell membranes (*3*). In such a matrix, ionic gradients would be maintained for prolonged periods at 0°C in the absence of a temperature-dependent change in ionic preference. In addition, accumulation against a concentration gradient of other solutes, such as sugar and amino acids, would be expected. Under certain conditions the steady-state concentration of glucose in the intracellular water of frog muscle at 0°C is about 120 percent of the external concentration (*5*). My data indicate that much higher amino acid gradients can be achieved at this temperature.

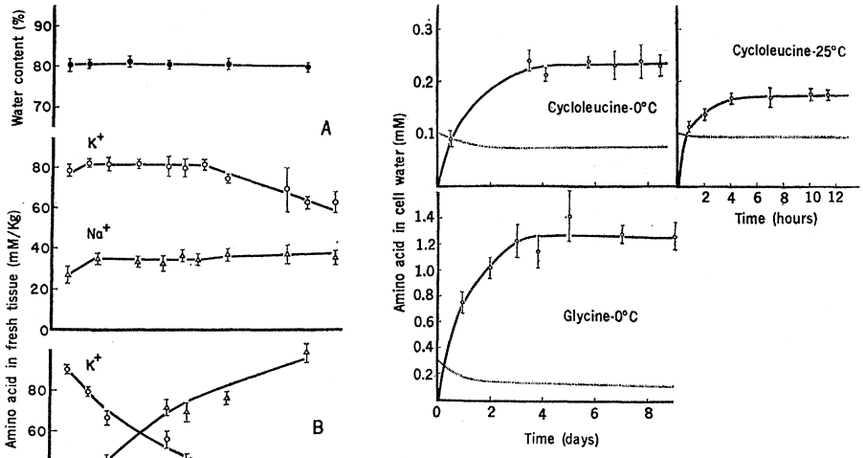

Fig. 1 (left). Water, Na^+, and K^+ content of frog muscles incubated for varying lengths of time in RP at 0°C. Distance between bars, two standard errors of the mean. (A) RP with 2.5 m*M* K^+. (B) Potassium-free RP. Fig. 2 (right). Time course of [^{14}C]amino acid entry into frog muscles. Dotted line, external amino acid concentration. Amino acid concentration in the cell water was calculated from the measured tissue content using an extracellular space of 12 percent, and a tissue water content of 80 percent. Final distribution ratios: glycine, 9.9; cycloleucine at 0°C, 2.8; cycloleucine at 25°C, 1.7.

Early workers (*12*) suggested that amino acid accumulation is due to adsorption on specific sites within the cytoplasm. Interpretation of my finding of increased cycloleucine uptake with decreased temperature is quite straightforward on the basis of this hypothesis: The equilibrium concentration of intracellular amino acid is a function of the number of adsorption sites and the affinity of the amino acid for these sites. As long as the cellular proteins maintain their "native" conformation, the number of adsorption sites will not change with temperature. An increase in binding with decreasing temperature would result from the increased affinity expected on thermodynamic grounds for a simple adsorption reaction (*13*).

Arguments against the binding hypothesis for amino acid accumulation have been summarized (*8, 14*). However, the data presented here suggest that these arguments should be reconsidered in an attempt to answer the question: To what extent is amino acid accumulation due to adsorption to specific sites on cell membranes or within the cytoplasm (or both)?

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