Effect of Proline on Lactate Dehydrogenase Activity: Testing the Generality and Scope of the Compatibility Paradigm*

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ABSTRACT The k_{cat} and K_m kinetic parameters of the labile enzyme rabbit muscle lactic dehydrogenase were determined as a function of the concentration of proline, a solute (osmolyte) accumulated in the cells of many organisms to protect them against environmental stresses. Proline is believed to protect against the stress(es) without altering the functional activity of cellular macromolecules, a property defining it as a "compatible osmolyte." In the range of 0–2 M proline, k_{cat} and K_m values for both substrates are essentially unchanged, but between 2 M and 4 M proline, k_{cat} decreases by a factor of 3 to 4, whereas K_m values are only modestly changed, if at all. These results are consistent with the proposal that compatible osmolytes do not affect functional activity, that the property of compatibility expressed by such osmolytes is generic without regard to the evolutionary history of the protein, and that the organic osmolyte concentration range over which compatibility is exhibited is extensive. In short, the results are in full accord with the principal hypothesis of "compatible osmolytes" in detail and scope.

INTRODUCTION

A common feature of organisms that have adapted to water-stress conditions is the accumulation of small organic solutes (Yancey et al., 1982). These solutes are often referred to as organic osmolytes and are believed to be of central importance in permitting the organism to live under water-stress conditions (Brown and Simpson, 1972; Pollard and Wyn Jones, 1979; Stewart and Lee, 1974; Yancey et al., 1982). A basic hypothesis of biological adaptation is that the osmolytes stabilize proteins and other macromolecular cell components against stress, but have little or no effect on the functional activity of those macromolecules (Pollard and Wyn Jones, 1979; Stewart and Lee, 1974; Yancey et al., 1982). That is, through evolution, such organisms have solved the problem of adapting to an environmental stress that could have affected the stability and biological activity of many of their macromolecules, and the solution to the problem seems to have involved the uniform stabilization of macromolecules, without affecting macromolecular function. Osmolytes that afford protection against water stress without affecting protein function are defined as "compatible solutes" (Brown and Simpson, 1972).

Examples of amino acids that are compatible osmolytes include glycine, proline, alanine, taurine, and β-alanine (Bowlus and Somero, 1979). These amino acids accumulate at levels of tens to hundreds of millimoles per kilogram of cell water in organisms such as those mentioned above (Yancey et al., 1982). Because a significant amount of cell water is associated with cell components and does not participate as solvent water, it is likely that the actual cellular concentration of these compatible osmolytes can be higher than might first appear, especially because increased osmotic tension external to some organisms is known to reduce the cytoplasmic volume (Cayley et al., 1991).

Reasonably high concentrations of proline occur in such water-stressed organisms as salt-tolerant bacteria, particular vascular plants, and marine invertebrates (Yancey et al., 1982). The diversity of organisms that concentrate this amino acid for the purpose of adaptation suggests that proline accumulation is an example of convergent evolution, providing a simple, efficient, and general solution in adaptation to a water-stress environment (Yancey et al., 1982). The concept of the natural selection of solutes that stabilize proteins without affecting biological activity is of major interest for understanding interrelationships between solution properties and protein structure/function.

In this communication we look at the effects of proline concentration on the k_{cat} and K_m parameters of rabbit muscle lactic dehydrogenase. By using an enzyme that is labile and unaccustomed to the presence of osmolytes, we aim to investigate the hypothesis that a typical compatible osmolyte (e.g., proline) is generic in its effect of having little or no influence on the functional activity of proteins (Carpenter and Crowe, 1988; Yancey et al., 1982).

Only a modest number of enzymes have been investigated with respect to the question of whether compatible osmolytes are innocuous in their effects on the biological activity of proteins, and although the kinetic parameters of most of the enzymes studied appear to be unaffected, exceptions have been reported (Bowlus and Somero, 1979; Brown and Simpson, 1972; Stewart and Lee, 1974; Yancey et al., 1982). In general, the maximum concentration of proline used in determining the effects of this compatible osmolyte on protein function is on the order of 1 M (Bowlus and Somero, 1979; Yancey et al., 1982). By observing the

Received for publication 12 June 1996 and in final form 7 August 1996.

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*The authors wish to acknowledge the enormous contribution of Prof. Timasheff to the understanding of solvent effects on proteins. His approach to science and insightful contributions have served as a great source of inspiration and stimulation to us.

0006-3495/96/10/2117/06 $2.00

effects of osmolyte concentration on k_{cat} and K_m over a much wider range of proline concentration than has previously been studied, we seek to establish the osmolyte concentration range over which the concept of "compatibility" fails or is attenuated.

MATERIALS AND METHODS

Lactate dehydrogenase (LDH) was purchased from Worthington Biochemical Corporation, proline was from CalBiochem, ultrapure grade Tris was from Aldrich. NADH, sodium pyruvate, and bovine serum albumin were from Sigma. These compounds were used without further purification.

LDH assays were carried out at 25°C, pH 7.3, in the absence or presence of different proline concentrations ranging from 1 to 4 M. LDH solution was prepared in 0.20 M Tris-HCl (with or without proline) along with 1 mg/ml bovine serum albumin and was kept on ice. The concentration of LDH was determined from absorbance at 280 nm (1.13 mg/ml/OD; supplied by Worthington). Molar absorptivities of NADH in the presence of up to 4 M proline concentrations were determined and found to be identical with the absorptivity in the absence of proline. Assays of LDH-catalyzed reactions were evaluated by following the oxidation of NADH at 340 nm, using a molar absorptivity of 6.22×10^3 M^{-1}-cm^{-1} to convert rates to a molar concentration basis. The LDH assays were performed by adding 2.8 ml of Tris-HCl buffer (with or without proline) to the sample cuvette and zeroing the baseline at 340 nm. NADH (100 μl) of different concentrations (in the presence or absence of proline) was added to the assay mixture. This was followed by the addition of 100 μl of sodium pyruvate (of different concentrations, with or without proline) and 100 μl of LDH stock solution containing the same proline concentration as the assay. The LDH stock (with or without proline) was prepared in the cold at about 0.6 μg/ml with Tris-HCl buffer containing 1 mg/ml bovine serum albumin. An aliquot of this stock was equilibrated to room temperature for 1–2 min just before the assay. The reaction was monitored by recording the change in absorbance at 340 nm as a function of time. The initial linear rates (OD/min) were converted to NADH concentration units and divided by the mass of LDH used in the assay (mmol/min/mg).

RESULTS

The traditional means of evaluating kinetic parameters of enzyme-catalyzed reactions makes use of linearized forms of rate equations. Fig. 1 shows (linear least squares) reciprocal plots (primary plots) of velocities with NADH concentration at various fixed concentrations of pyruvate. In agreement with previous studies, reciprocal plots of velocity with pyruvate concentration at fixed concentrations of NADH are found to be linear. Through extensive studies, Zewe and Fromm obtained kinetic data similar to those shown in Fig. 1 and concluded that their data were consistent with a modified form of the Theorell-Chance mechanism (Zewe and Fromm, 1965). The rate expression for this mechanism in the absence of products is given in Eq. 1:

$$1/v = (1/k_{cat}) * (1 + K_{NADH}/[NADH] + K_{pyr}/[pyr] + K_{NADHpyr}/[NADH][pyr)] \quad (1a)$$

$$v = k_{cat}[NADH][pyr]/([NADH][pyr] + K_{NADH}[pyr] + K_{pyr}[NADH] + K_{NADHpyr}) \quad (1b)$$

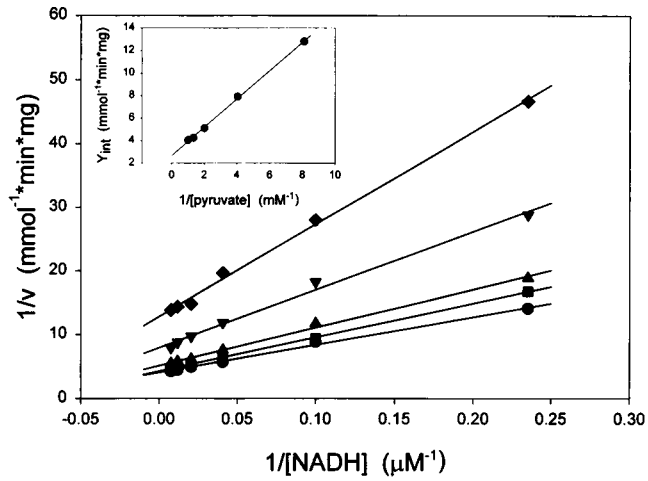

FIGURE 1 Lineweaver-Burk (primary plot) of LDH activity at pH 7.3, 25°C in the absence of proline. The initial velocity (v) was measured as a function of NADH concentration at pyruvate concentrations of 986.3 μm (●), 739.7 μm (■), 493.2 μm (▲), 246.6 μm (▼), and 123.3 μm (◆). The lines are linear fits of the data at each pyruvate concentration. The intercepts of these lines on the y axis (Y_{int}) are plotted in the inset as a function of 1/[pyruvate] (secondary plot). The solid line is a linear fit to the data.

According to the modified Theorell-Chance mechanism proposed by Zewe and Fromm, the slopes and intercepts of $1/v$ versus 1/[NADH] at various fixed concentration of pyruvate, as shown in Fig. 1, give rise to the relationships given in Eq. 2 (Zewe and Fromm, 1965). These relationships suggest plots (secondary plots) of intercepts or slopes versus 1/[pyr] will be linear, and from these plots values of K_{NADH}, K_{pyr}, $K_{NADH\ pyr}$, and k_{cat} may be extracted:

$$\text{Intercepts} = 1/k_{cat} + K_{pyr}/k_{cat}[pyr] \quad (2a)$$

$$\text{Slopes} = K_{NADH}/k_{cat} + K_{NADHpyr}/k_{cat}[pyr]. \quad (2b)$$

A representative linear least-squares fit of intercepts versus 1/[pyr] is given in the inset of Fig. 1.

Alternatively for primary and secondary plots, a more statistically appropriate means of evaluating kinetic parameters (K_{NADH}, K_{pyr}, $K_{NADH\ pyr}$, and k_{cat}) is to fit simultaneously velocity versus [pyruvate] data at fixed concentrations of NADH, using nonlinear least-squares analyses. A comparison of the kinetic parameters evaluated by the two methods, along with values reported by Zewe and Fromm (1965), is given in Table 1.

Evaluation of rate data for LDH-catalyzed reduction of pyruvate in the presence of 1, 2, 3, and 4 M proline were obtained and analyzed by nonlinear least-squares fitting of the velocity data versus [pyruvate] and [NADH] using Eq. 1b. The fitted results of simultaneous analysis for experiments performed in the presence of 4 M proline are shown in Fig. 2. The quality of data and fits in 4 M proline are representative of data obtained at each concentration of proline.

The kinetic parameters evaluated using global nonlinear least-squares analyses are shown in Figs. 3 and 4, with the

TABLE 1 Kinetic parameters for rabbit muscle LDH

	k_{cat}	$K_{m_{pyr}}$ (μM)	$K_{m_{NADH}}$ (μM)	$K_{m_{pyr\text{-}NADH}}$ (μM)^2
LLS*	0.375	471	11.9	5290
NLLS#	0.366	417	10.5	6450
Zewe and Fromm§	—	209	7.43	1140

*LLS, linear least-squares analysis of primary (Eq. 1a) and secondary (Eq. 2a) plots. This work, 25°C, pH 7.3.
#NLLS, nonlinear least-squares analysis using Eq. 1b. This work, 25°C, pH 7.3.
§Zewe and Fromm (1965), 28°C, pH 7.15.

dependencies of the Michaelis constants, K_{NADH}, K_{pyr}, and $K_{NADH\ pyr}$ on proline concentration shown in Fig. 3, and the variation of k_{cat} with proline concentration is given in Fig. 4.

DISCUSSION

The double-reciprocal and secondary plots for evaluating k_{cat} and K_m shown in Fig. 1 illustrate the traditional means of numerically evaluating K_m and k_{cat} enzyme kinetic constants. Although they are not as visually illustrative (see Fig. 2), nonlinear least-squares fits of kinetic data to Eq. 1b are preferred because they obviate problems with error analyses introduced when the double-reciprocal form of the rate equation is used (Johnson and Frasier, 1985). Table 1 compares kinetic parameters evaluated using nonlinear and linear least-squares methods, along with kinetic parameters reported by Zewe and Fromm (1965) under similar pH and temperature conditions. Although many of the kinetic parameters of Zewe and Fromm are roughly comparable to ones we obtained, there are parameters that differ significantly more than might be expected, based on the modest differences between experimental conditions. However, Zewe and Fromm note that their kinetic results differ from those of Thompson and attribute the differences to possible differences in rabbit muscle isozyme forms in the two studies (Thomson et al., 1964; Zewe and Fromm, 1965). Different ratios of isozymes may also explain the differences between our kinetic parameters and those of Zewe and Fromm. Our goal in this study is to determine how kinetic parameters change with proline concentration, and

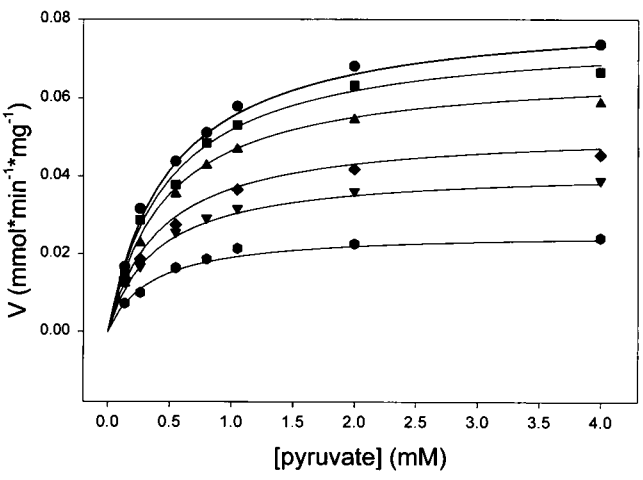

FIGURE 2 Hyperbolic plot of LDH activity at pH 7.3, 25°C in the presence of 4 M proline. The initial velocity (v) was plotted as a function of pyruvate concentration at NADH concentrations of 7.68, 16.3, 25.1, 51.4, 87.1, and 136.3 μM. The solid lines are nonlinear least-squares best fits of the data to Eq. 1b, giving the kinetic constants listed in Table 1.

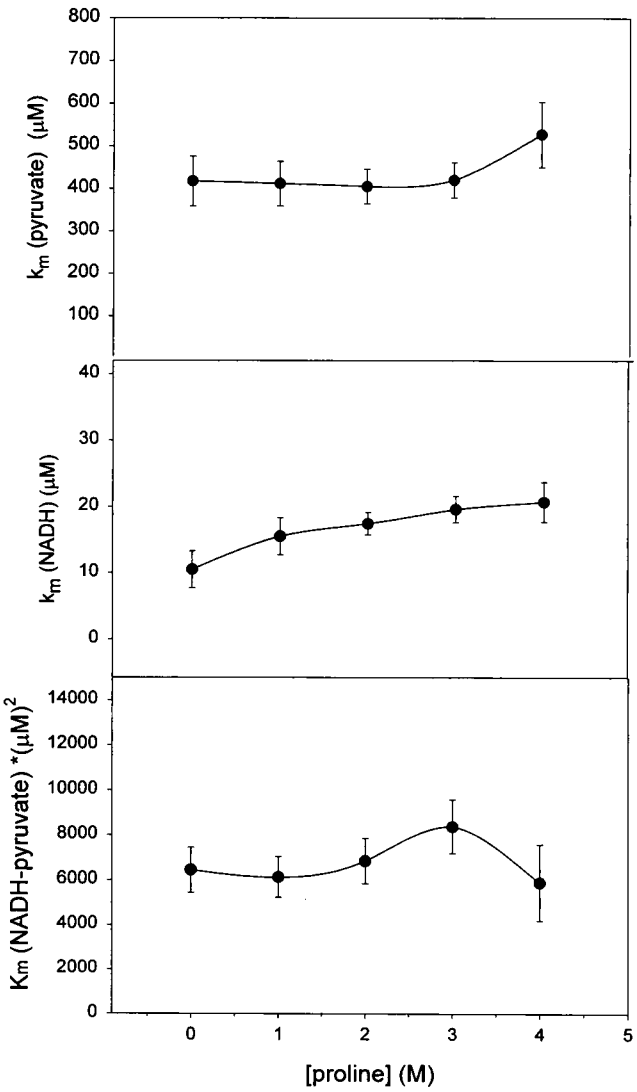

FIGURE 3 Effects of proline on K_m values for pyruvate (*top*), NADH (*middle*), and NADH-pyruvate (*bottom*). The solid lines are drawn simply to pass through the points; they have no theoretical significance. Bars represent errors obtained through nonlinear least-squares fits of the data.

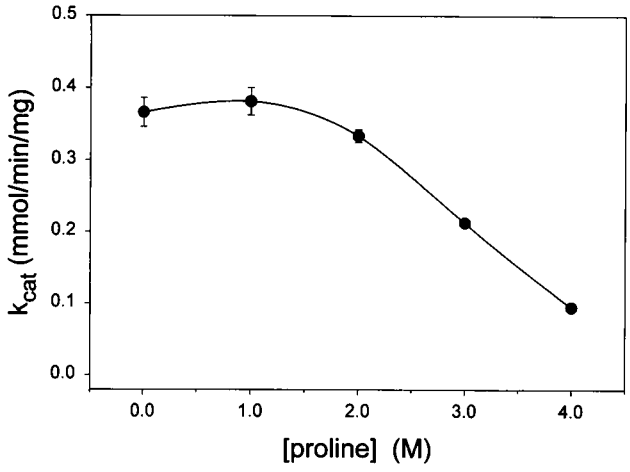

FIGURE 4 Effects of proline on the catalytic rate constant, k_{cat}. Bars represent errors obtained through nonlinear least-squares fits of the data to Eq. 1b. Errors of fitting at 3 and 4 M proline are within the size of the symbol.

this can be accomplished regardless of differences in absolute values from different studies.

Proline is known to protect plants from stresses such as heat, cold, salt, and dehydration, and microorganisms are protected from the osmotic stress of high salt concentration by accumulation of this amino acid. For an osmolyte to provide a selective advantage in biological adaptation to a stress, two problems must be solved: 1) the osmolytes must alleviate the effects of the stress without adversely affecting the stability of the macromolecules in the cells of the organism, and 2) the presence of the osmolyte cannot substantially affect the functional activity of the macromolecules and other cellular components. The latter condition is just as important as the former, because osmolyte protection would be of little selective advantage if functional activity was not preserved to maintain the intricate control of the metabolic pathways necessary to sustain life.

The property of not interfering with the functional activity of proteins is the defining characteristic of a class of osmolytes known as "compatible osmolytes" (Bowlus and Somero, 1979; Brown and Simpson, 1972). Because the property of being innocuous to enzyme function is thought to be uniformly exhibited in organisms that adopted compatible osmolytes evolutionarily (Yancey et al., 1982), it is assumed that compatible osmolytes will exhibit the property in the presence of any and all proteins, regardless of whether those proteins evolved in the presence of the osmolytes. The present study was undertaken to determine the effect of the compatible osmolyte proline on the kinetic parameters of rabbit muscle LDH. This protein does not experience substantive concentrations of proline in its normal environment, and it serves as a model for testing the extent to which the property of compatibility is independent of the evolutionary history of the protein. The fact that this protein is labile also adds to the stringency of the test (Carpenter and Crowe, 1988).

K_m parameters are expected to be affected by solvent because they are composed of (bimolecular) rate constants involving intermolecular events (association of enzyme with substrate) exposed to solvent; k_{cat}, on the other hand, is composed of molecular events within the protective confines of the enzyme (unimolecular rate constants) and should be much less affected by solvent. A high concentration of proline might be expected to affect the association of substrate with enzyme in any one of several ways, through solvation effects on substrates or enzyme active sites, by means of effects on the thermodynamic activity of substrates or enzyme (Bowlus and Somero, 1979; Cayley et al., 1992; Schoberte, 1977), or by effects on the physical properties of the solution (Bolen and Fisher, 1969). What is observed in Figs. 3 and 4 is exactly opposite what is expected, namely, K_m parameters for LDH-catalyzed reactions are relatively unaffected (within error) over the proline concentration range of 0–4 M proline, whereas k_{cat} decreases by three- to fourfold as the osmolyte concentration changes from 2 to 4 molar proline. On the basis of the rate constants that comprise k_{cat}, the influence of proline suggests that the enzyme species most affected by the presence of 2–4 M proline are one or more enzyme-product transition states. Alternatively, because an essential step in the catalytic sequence of the modified Theorell-Chance mechanism is an isomerization of the enzyme-oxidized coenzyme complex (Zewe and Fromm, 1965), and this rate constant is also incorporated in the k_{cat} parameter, the decrease in k_{cat} may be due to the effect of proline on the isomerization event. In either case, it is important to note that proline apparently has little or no effect on the solvation properties of substrates and enzyme active sites, because there is very little effect on K_m values, at least to an extent that would compromise the kinetics or binding of enzyme to substrate. The behavior exhibited in Figs. 3 and 4 is consistent with the premise of "compatible osmolytes," in that the lack of effect on bimolecular events in catalysis is essential if a compatible osmolyte is to avoid altering the kinetics of any enzyme.

The kinetic parameters of LDH do not begin to deviate substantially from the concept of compatibility until proline concentrations exceed 2 M. It is extremely unusual for a solute in the range of 0–2 M to have no affect on enzyme activity (Bolen and Fisher, 1969; Castaneda-Agulló and Del Castillo, 1959a,b; Sluyterman, 1967), and the possibility that proline may be innocuous to the activity of any enzyme is indeed remarkable. With respect to the biological consequences of these observations, two issues emerge: one having to do with the effective concentration range over which compatibility applies, and the other dealing with identifying properties of a solute that permit it to be innocuous toward protein function. Both of these issues address the concentration limits to which the "compatibility" phenomenon can be expressed.

The ability of rabbit muscle LDH to function perfectly well in 2 M proline is observed to be well above the tens to hundreds millimolar proline normally observed in water-stressed organisms (Bowlus and Somero, 1979; Yancey et

al., 1982). Thus, if rabbit muscle LDH could be considered typical in its response to proline, the 0–2 M concentration range would afford considerable reserve in the ability of the organism to tolerate the stress. Because the concentrations of proline evaluated in organisms are expressed on a basis of total cell water (Yancey et al., 1982) without allowance for compartmentation, the amount of water that participates as solvent, and other factors, it may be that the local concentration of proline in a particular location in the cell is higher than the average values normally reported. At this point we can only say that the proline concentration LDH can tolerate without affecting function is considerably higher than the (average) concentrations found in water-stressed organisms, suggesting a significant range over which the compatibility paradigm can operate with this enzyme.

The issue of which properties of proline permit it to be innocuous toward protein function is key to understanding the basis of compatibility. From the elegant studies of Timasheff and co-workers, it is known that compatible osmolytes exhibit preferential hydration and that this property is responsible for the stabilizing effect compatible osmolytes have on proteins (Arakawa et al., 1990; Arakawa and Timasheff, 1982a,b, 1983, 1984a,b,c, 1985; Lee and Lee, 1981; Lee and Timasheff, 1981; Timasheff, 1992a,b, 1993). However, this property, although it is common to all compatible osmolytes, does not explain why these solutes are innocuous in their effects on the biological function of the protein, for there are examples of solutes that stabilize proteins by preferential hydration but significantly affect the function of the protein (Chadalavada et al., 1994). It is well documented in the literature that nonosmolyte solutes in the submolar to 1 M concentration range cause marked changes in enzyme kinetic parameters. These changes in enzyme activity are due to a variety of causes, including solute interaction with the protein or substrates, osmotic stress, or changes in such solution properties as dielectric constant, surface tension, viscosity, water activity, etc. (Bolen and Fisher, 1969; Bowlus and Somero, 1979; Castaneda-Agulló and Del Castillo, 1959a,b; Faller and Sturtevant, 1966; Rand et al., 1993). It is extremely difficult for a solute in the 0–2 M range not to alter one or more of these solution properties and thereby alter kinetic properties. In light of data on the physical properties of proline solutions, one possibility is that compatible osmolytes may be a highly select group of solutes that do not interact with substrates or protein, or substantively alter the physical and chemical properties of aqueous solutions at concentrations in the 1–2 M range. We are pursuing this line of investigation.

The results presented here are consistent with the proposal that proline in the range of 0–2 M has essentially no effect on k_{cat} and K_m values of rabbit muscle LDH. These observations are in accord with the hypothesis that compatible osmolytes such as proline do not affect the functional activity of protein, that the effect occurs without regard to the evolutionary history of the protein, and that the osmolyte concentration range over which compatibility is exhibited is extensive. Clearly, more enzymes that have not evolved in the presence of compatible osmolytes must be studied in the presence of compatible osmolytes before statements concerning the generality of the phenomenon can be established. The use of a labile enzyme like LDH provides a reasonable test of the essentials of the compatibility proposal, and the fact that 2 M proline is innocuous to the functional activity of the enzyme is in concord with the proposal.

Supported by U.S. Public Health Research grant GM49760.

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