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Counteraction of Urea Destabilization of Protein Structure by Methylamine Osmoregulatory Compounds of Elasmobranch Fishes

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Intracellular fluids of marine elasmobranchs (sharks, skates and rays), holocephalans and the coelacanth contain urea at concentrations averaging 0.4M, high enough to significantly affect the structural and functional properties of many proteins. Also present in the cells of these fishes are a family of methylamine compounds, largely trimethylamine *N*-oxide with some betaine and sarcosine, and certain free amino acids, mainly β-alanine and taurine, whose total concentration is approx. 0.2M. These methylamine compounds and amino acids have been found to be effective stabilizers of protein structure, and, at a 1:2 molar concentration ratio of these compounds to urea, perturbations of protein structure by urea are largely or fully offset. These counteracting effects of solutes on proteins are seen for: (1) thermal stability of protein secondary and tertiary structure (bovine ribonuclease); (2) the rate and extent of enzyme renaturation after acid denaturation (rabbit and shark lactate dehydrogenases); and (3) the reactivity of thiol groups of an enzyme (bovine glutamate dehydrogenase). Attaining osmotic equilibrium with seawater by these fishes has thus involved the selective accumulation of certain nitrogenous metabolites that individually have significant effects on protein structure, but that have virtually no net effects on proteins when these solutes are present at elasmobranch physiological concentrations. These experiments indicate that evolutionary changes in intracellular solute compositions as well as in protein amino acid sequences can have important roles in intracellular protein function.

Studies of solute effects on a wide variety of macromolecular structural and functional properties have revealed a high consistency or ranking among certain solutes in terms of their stabilizing or destabilizing influences on these properties. For example, cations and anions of neutral salts display a characteristic ranking, the Hofmeister or lyotropic series, in their effects on protein-structural stability, protein solubility, DNA 'melting' temperature, enzyme activity and other macromolecular phenomena (von Hippel & Schleich, 1969). As another example, urea and related compounds invariably act as destabilizers of these diverse aspects of macromolecules (Gordon & Jencks, 1963; von Hippel & Schleich, 1969). In view of these sharp and consistent differences among solutes in their effects on proteins and nucleic acids, it is appropriate to ask whether such effects are evolutionally important in determining which solutes are selectively accumulated in, or excluded from, the cellular fluids of organisms. This question is especially germane in the case of organisms having very high concentrations of solutes in their cells, e.g., marine invertebrates and marine elasmobranchs (sharks, skates and rays), which have intracellular osmolalities equal to that of seawater (Prosser, 1973; Pang *et al.*, 1977). If solute–macromolecule interactions are important in cellular function and evolution, clearest evidence of this importance should be found in such organisms.

Of particular interest in many marine iso-osmotic organisms is the use of high concentrations of nitrogenous metabolites as osmotic agents. Some of these compounds may have marked effects on macromolecular systems *in vivo*, especially in the case of the marine elasmobranchs, holocephalans and the coelacanth, which maintain concentrations of urea (averaging 0.4M) high enough to perturb the structures of many proteins and the functions of many enzymes (Rajagopalan *et al.*, 1961; see references in Yancey & Somero, 1978). However, these fishes also accumulate in their cells fairly high concentrations of methylamine compounds, largely

Abbreviations used: Nbf-Cl, 4-chloro-7-nitrobenzofurazan; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK').

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trimethylamine *N*-oxide (*NN*-dimethylmethanamine *N*-oxide) with lesser amounts of betaine (*N*-trimethylglycine; 1-carboxy-*NNN*-trimethylmethanaminium hydroxide inner salt) and sarcosine (*N*-methylglycine). and certain free amino acids, mainly β-alanine (3-aminopropanoic acid) and taurine (2-aminoethanesulphonic acid). These methylamine compounds and amino acids are found at concentrations totalling 0.2M average, or about half the concentration of urea (Pang *et al.*, 1977; Boyd *et al.*, 1977; Robertson, 1975). The structures of these compounds resemble the stabilizing ions of the Hofmeister series, e.g., the tetramethylammonium ion, suggesting that these osmoregulatory compounds may be able to exert stabilizing influences on macromolecules and thus offset the destabilizing effects of urea (Clark & Zounes, 1977; Yancey, 1978).

In an initial investigation of this possibility, we have shown that, indeed, the principal methylamine osmoregulatory solutes, namely trimethylamine *N*-oxide, betaine and sarcosine, have effects opposite to those of the urea on the kinetic properties ($V_{max.}$ and K_m) of many enzymes (Yancey, 1978). When the methylamine compounds and urea are present at elasmobranch physiological concentrations or ratios (about 1:2 molar ratio), their opposing effects counterbalance in most cases. Like the effects of urea and ions of the Hofmeister series, the urea-antagonistic effects of these methylamine solutes are not restricted to elasmobranch enzymes, but are independent of the species source of enzyme (Yancey, 1978). This supports the idea that these compounds, like the structurally similar stabilizing ions of the Hofmeister series, are general macromolecular stabilizers. In the present study we have further tested this hypothesis by using mammalian and elasmobranch enzymes to determine the effects of these osmoregulatory solutes on protein-structural phenomena, including enzyme thermal stability, rate of enzyme thiol-group labelling and renaturation after denaturation.

Experimental

Three urea-sensitive protein-structural phenomena were studied to determine the extent to which methylamine and certain other osmoregulatory solutes could offset urea destabilization.

Thermal denaturation of ribonuclease

Ribonuclease A (EC 3.1.4.22) from bovine pancreas (five-times-recrystallized; Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved (3mg/ml) in 37.5mM-potassium phosphate buffer, pH6.7, with 225mM-KCl. Water or a solution of nitrogenous compounds (1ml; adjusted to pH6.7 with small amounts of KOH or HCl) was then added to 2ml of this ribonuclease solution. The A_{287} of this final mixture, which indicates the extent of exposure of tyrosine residues to the solvent (Hermans & Scheraga, 1961), was monitored in sealed quartz cuvettes in a water-jacketed four-sample Gilford spectrophotometer; the temperature was controlled by a circulating-water bath. Three cuvettes held samples; the fourth held a thermoprobe. Absorbance was recorded at 1°C intervals after the samples had been allowed to equilibrate for 15min at each temperature. The 'melting' temperature, T_m, was taken to be that at which the A_{287} was halfway between the absorbance at 40°C (native ribonuclease) and the lowest absorbance recorded between 67 and 70°C (denatured protein). As a control, the absorbance of a solution of tyrosine was measured as a function of temperature and found to be negligibly affected by the various solutes.

Renaturation of lactate dehydrogenase

Fully purified skeletal-muscle lactate dehydrogenases (L-lactate–NAD^+ oxidoreductase, EC 1.1.1.27; the major skeletal muscle isoenzyme, designated 'M_4 lactate dehydrogenase') were obtained from an elasmobranch fish, the white shark (*Carcharodon carcharias*) (the enzyme was generously provided by the laboratory of Dr. N. O. Kaplan, Biology Department, University of California, San Diego, CA, U.S.A.), and from a mammal, the rabbit (enzyme purchased from Sigma). The procedures for renaturation were those described by Rudolph *et al.* (1977). Each enzyme was dialysed against 0.2M-potassium phosphate buffer, pH7.2, at 4°C, with 0.1mM-EDTA and 0.1mM-dithiothreitol. The dialysed enzymes were then diluted to concentrations of 0.9, 5 or 8μg/ml as determined by absorption coefficients (Pesce *et al.*, 1967). To a 0.4ml portion of enzyme was added 0.1ml of a solution containing 1M-glycine/H_3PO_4 (pH2.3 at room temperature), 5mM-EDTA and 5mM-dithiothreitol. Each sample was allowed to denature in this acidic medium, on ice, for precisely 15min, and then was diluted 1:10 (at 20 or 37°C) with 2ml of water or solution (pH7.2) of nitrogenous solutes and 2.5ml of 0.4M-potassium phosphate buffer, pH7.6, containing 1.5mM-NAD^+ and 1mM-dithiothreitol (final pH 7.2). Renaturation was followed at either 20 or 37°C by assaying the activity (pyruvate reductase direction) of 50μl portions of the sample in 0.1M-potassium phosphate buffer, pH7.0, with 3mM-sodium pyruvate and 0.15mM-NADH, and 20μg of Tos-Phe-CH_2Cl-treated trypsin/ml [the last added to prevent renaturation in the cuvette during the assay (Chan *et al.*, 1973)]. Activity was determined from the decrease in A_{340}.

Reactivity of thiol groups of glutamate dehydrogenase

The reaction of Nbf-Cl with the thiol groups of purified bovine liver glutamate dehydrogenase

[L-glutamate–NAD(P)$^+$ oxidoreductase (deaminating); EC 1.4.1.3; Sigma] was monitored by increase in A_{240}, as described by Kapoor & Parfett (1977). The Nbf-Cl was dissolved in 100% ethanol (8.2 or 16.4mg/ml) and added in a small volume to the reaction mixture, consisting of glutamate dehydrogenase dissolved at various concentrations in either 50mM-Tris/HCl, pH7.8 at 22°C, or 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH7.8 at 37°C (pH adjusted with KOH), with 50mM-KCl, 0.75mM-NAD$^+$ and 0.1mM-EDTA. Concentrations of enzyme and Nbf-Cl are given in the Figure legends.

Chemicals

Solutions of nitrogenous compounds were prepared fresh daily. Urea solutions were treated with a mixed-bed ion-exchange resin (Amberlite MB-3) to remove cyanate and NH_4^+. Sarcosine (U.S. Biochemicals, Cincinatti, OH, U.S.A.), β-alanine, betaine, taurine, and tyrosine (Sigma) and trimethylamine *N*-oxide (Eastman Kodak Co., Rochester, NY, U.S.A.) were all reagent grade and were used without further purification. Tos-Phe-CH_2Cl-treated trypsin was obtained from Worthington Biochemicals, Freehold, NJ, U.S.A. Nbf-Cl, EDTA, dithiothreitol and buffer reagents were obtained from Sigma.

Results and Discussion

Thermal denaturation of ribonuclease

Bovine pancreatic ribonuclease has served as a classic model protein in studies of solute effects on protein-structural stability (von Hippel & Wong, 1965; von Hippel & Schleich, 1969). Neutral salts affect the 'melting' temperature (T_m) of ribonuclease according to their positions in the Hofmeister series, and urea effectively decreases T_m. To evaluate the effects of methylamine solutes and certain free amino acids on thermal stability of proteins we have therefore used this enzyme as a model, with standard ribonuclease heat-denaturation protocols used by other workers (e.g. Hermans & Sheraga, 1961).

The results in Figs. 1 and 2 show that trimethylamine *N*-oxide and β-alanine significantly elevate the T_m of ribonuclease; taurine, sarcosine and betaine also have slight to moderate stabilizing effects (Fig. 2). These structure-stabilizing effects appear to occur even at relatively low solute concentrations (0.1M). As for previously observed neutral-salt effects (von Hippel & Schleich, 1969), the influences of structure-stabilizing and structure-destabilizing solutes are additive; thus the stabilizers can counteract the effects of urea (Figs. 1 and 2). It is also noted that the size as well as the midpoint (T_m) of the transition is affected by solutes (Fig. 1): urea increases the total change in absorbance (as previously noted by von Hippel & Wong, 1965), whereas the stabilizers

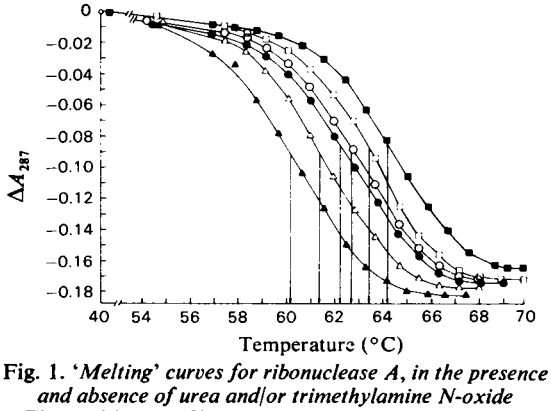

Fig. 1. *'Melting' curves for ribonuclease A, in the presence and absence of urea and/or trimethylamine N-oxide*
The melting profiles were determined as described in the Experimental section. The vertical arrows mark the 'melting' temperatures (T_m). ○, Control; □, 200mM-trimethylamine *N*-oxide; ■, 400mM-trimethylamine *N*-oxide; △, 400mM-urea; ▲, 800mM-urea; ●, 200mM-trimethylamine *N*-oxide plus 400mM-urea.

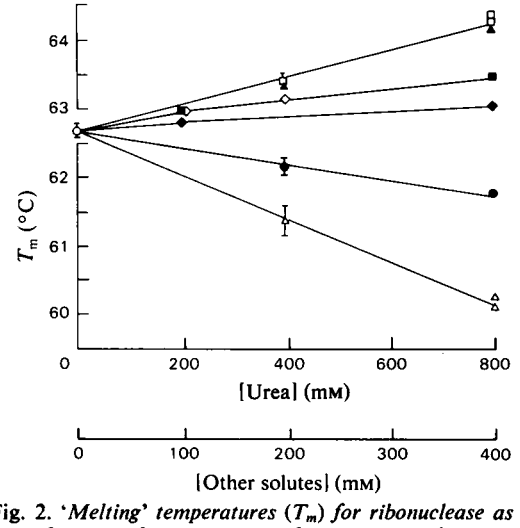

Fig. 2. *'Melting' temperatures (T_m) for ribonuclease as a function of concentration of nitrogenous solutes*
The abscissa represents two scales, one for urea concentrations and one for the total concentrations of the other nitrogenous solutes (the latter scale at a 1:2 ratio to that for urea). The error bars on several points represent S.D. values for five determinations. T_m values were determined as described in the Experimental section. ○, Control; △, urea; □, trimethylamine *N*-oxide; ■, betaine; ▲, β-alanine; ◇, sarcosine; ◆, taurine; ●, urea and trimethylamine *N*-oxide.

decrease the change (as also observed with neutral salts by von Hippel & Wong, 1965).

The ranking of these compounds in terms of stabilizing abilities is: trimethylamine *N*-oxide > β-alanine > betaine = sarcosine > taurine. The stabilizing effects of these compounds resemble the influences of structurally similar ions of the Hofmeister series on this same protein (von Hippel & Wong, 1965). Of these ions, tetramethylammonium bears structural similarities to trimethylamine *N*-oxide, betaine and sarcosine, ammonium to β-alanine and taurine, acetate to betaine, sarcosine and β-alanine, and sulphate to taurine. However, the similarities in effects on ribonuclease are only qualitative. Trimethylamine *N*-oxide, in particular, appears to be a better stabilizer than the tetramethylammonium ion and, indeed, elevates the T_m more than any of the zwitterionic solutes tested. This finding suggests that the lack of a full charge on the trimethylamine *N*-oxide nitrogen atom, or the presence of an oxygen in the molecule, increases the stabilizing ability of the methylammonium group.

Renaturation of lactate dehydrogenase

Denatured lactate dehydrogenase can be renatured at physiological temperatures and pH values to an apparently native state (Rudolph *et al.*, 1977), although quantitative recovery of original activity is generally not observed. The renaturation process involves both the refolding of secondary and tertiary structures of the subunit monomers and the reassociation of the monomers into the functional tetrameric enzyme (Rudolph & Jaenicke, 1976). The effects of the nitrogenous solutes on lactate dehydrogenase renaturation after acid denaturation are shown in Fig. 3(*a*) for shark lactate dehydrogenase at 20°C, and in Fig. 3(*b*) for the rabbit homologue at 37°C. The methylamine solutes are again found to favour the native enzyme, whereas urea has the expected opposite effect. The influences of urea and trimethylamine *N*-oxide are, in this case, balanced at a molar concentration ratio of about 2:1 (urea/trimethylamine *N*-oxide). The order of effectiveness in favouring renaturation is: trimethylamine *N*-oxide > betaine > sarcosine > β-alanine = taurine (the latter two having very weak effects), which, unlike the situation found with ribonuclease, is in the order of decreasing number of *N*-methyl groups. It should be noted that the effects of the solutes are less at higher protein concentrations, but are still signficant (Fig. 3*b*).

Although the effects of the solutes on these two homologous enzymes are qualitatively the same, there are quantitative differences. For the shark enzyme the effects appear to be almost entirely on the kinetics of renaturation (Fig. 3*a*). The extent of renaturation is only slightly dependent on solute

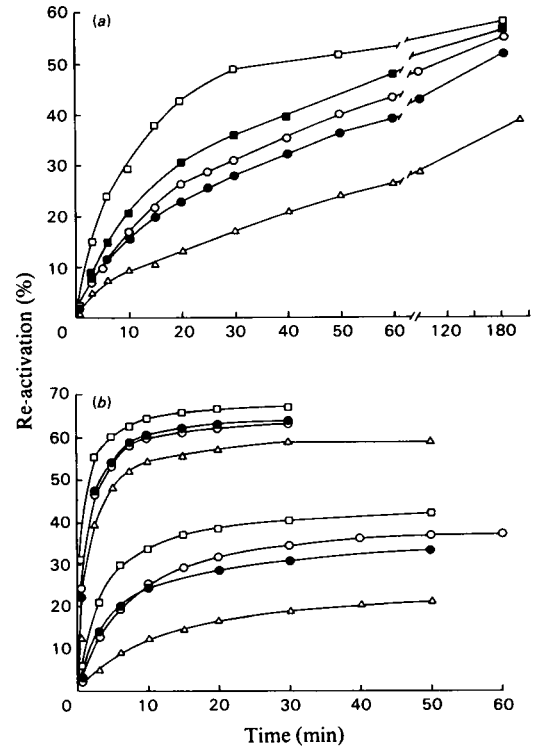

Fig. 3. *Recovery of activity of acid-denatured lactate dehydrogenases in the presence and absence of various nitrogenous solutes*

Renaturation occurred in the presence of NAD^+; see the Experimental section for details. ○, Control; □, 200mM-trimethylamine *N*-oxide; △, 400mM-urea; ●, 200mM-trimethylamine *N*-oxide plus 400mM-urea; ■, 100mM-betaine. (*a*) White-shark lactate dehydrogenase (M_4 isoenzyme), at 20°C, pH7.2, at a concentration of enzyme monomers of 150nM; (*b*) rabbit lactate dehydrogenase (M_4 isoenzyme), at 37°C, pH7.2, at two different monomer concentrations: upper four curves, 240nM; lower four curves, 28nM.

conditions. This latter conclusion was substantiated by plotting the data on double-reciprocal axes [1/(% renatured) against 1/time]. The extrapolated recovery at infinite time was only slightly decreased by urea and slightly increased by trimethylamine *N*-oxide and betaine. On the other hand, for the rabbit enzyme, both the extent of renaturation as well as the kinetics of the process were dependent on solute conditions (Fig. 3*b*). Similar differences between the two lactate dehydrogenases were observed under identical conditions of monomer concentration (150nM) and temperature (20°C) (results not shown). But for both enzymes the increase in renaturation rate in the presence of the urea antagonists resembles

the rate enhancement of ribonuclease renaturation by dibasic potassium phosphate and ammonium sulphate (Schaffer *et al.*, 1975), two structure-stabilizing salts.

Reactivity of thiol groups of glutamate dehydrogenase

A third approach to the question of urea-antagonistic effects on proteins involved the reaction of enzyme (glutamate dehydrogenase) thiol groups with the reagent Nbf-Cl. Kapoor & Parfett (1977) have demonstrated that this reagent is an excellent probe for examining the conformation of bovine liver glutamate dehydrogenase in solution. The reactivity of Nbf-Cl with thiol groups of this enzyme has been directly correlated with structural stability, enzymic activity and conformational state (as measured by c.d.) (Kapoor & Parfett, 1977).

Under conditions similar to those used by Kapoor & Parfett (1977) (Tris/HCl buffer, 22°C), the reactions of Nbf-Cl with enzyme shown in Fig. 4 were obtained. Again, at a 2:1 molar ratio of urea/trimethylamine *N*-oxide, counterbalancing effects of these two solutes are found. Only the kinetics of the reaction are affected by the solutes; if allowed to continue to completion, all four reactions attain the same absorbance, i.e., the number of thiol groups reacted with Nbf-Cl.

This type of study was repeated under more physiological conditions for this mammalian enzyme, namely 37°C and a higher ionic strength. Under these conditions the reaction of Nbf-Cl with Tris is substantial, and a less-reactive buffer cation, Hepes, was used. Even with the latter, however, some reaction with Nbf-Cl is observed (Fig. 5*a*), and must be subtracted from the absorbance change of the enzyme reaction with Nbf-Cl. Again, opposite and counteracting effects of urea and trimethylamine *N*-oxide are observed (Fig. 5*b*), with a counterbalance occurring at a 2:1 molar ratio of urea/trimethylamine *N*-oxide (Nbf-Cl reacts somewhat with charged amino groups, so that betaine, sarcosine, taurine and β-alanine could not be used in these experiments). Thus again trimethylamine *N*-oxide appears to be a macromolecular stabilizer, in this case apparently causing a shift in an equilibrium among conformational states of glutamate dehydrogenase towards more stable state(s). Urea, as expected, shows an opposite, destabilizing effect. As a control, reduced glutathione was tested with Nbf-Cl; this reaction was

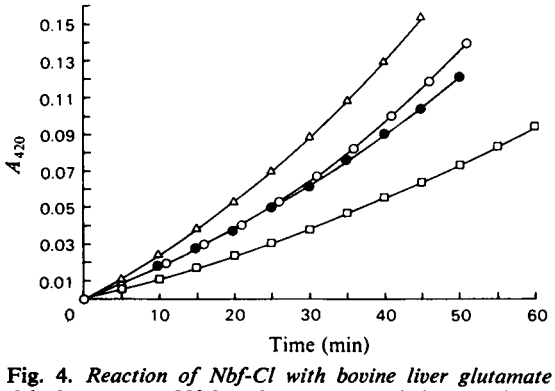

Fig. 4. *Reaction of Nbf-Cl with bovine liver glutamate dehydrogenase at 22°C in the presence and absence of urea and/or trimethylamine N-oxide*

Tris/HCl buffer was used with the enzyme at 0.2mg/ml and Nbf-Cl at 0.775mM. Curves are corrected for a very slight reaction of Nbf-Cl with buffer and the added solutes. ○, Control; □, 200mM-trimethylamine *N*-oxide; △, 400mM-urea; ●, 200mM-trimethylamine *N*-oxide plus 400mM-urea.

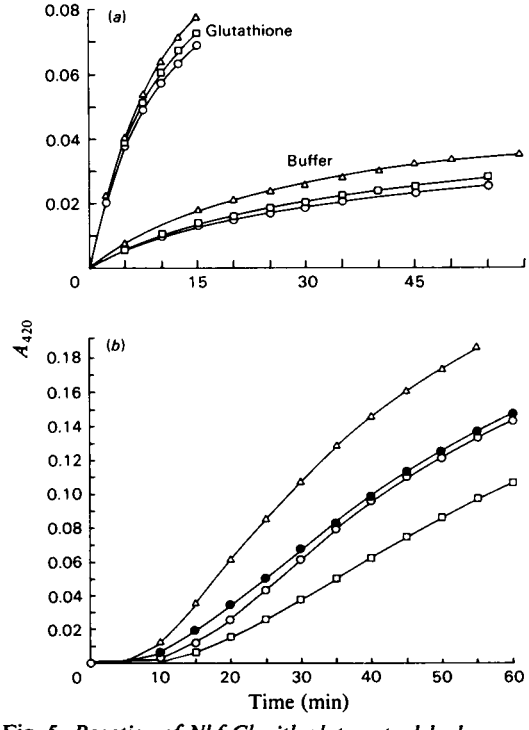

Fig. 5. *Reaction of Nbf-Cl with glutamate dehydrogenase, reduced glutathione and buffer at 37°C in the presence and absence of urea and/or trimethylamine N-oxide*

Hepes buffer was used, also containing KCl, NAD^+ and EDTA (see the Experimental section). ○, Control; □, 200mM-trimethylamine *N*-oxide; △, 400mM-urea; ●, 200mM-trimethylamine *N*-oxide plus 400mM-urea. (*a*) Nbf-Cl (5μM) with reduced glutathione (5μM) (three upper curves), and Nbf-Cl (0.338mM) with buffer system alone (three lower curves); (*b*) Nbf-Cl (0.338mM) with enzyme (0.04mg/ml), with lines corrected for the Nbf-Cl reaction with the buffer system and added solutes.

virtually unaffected by the solutes (Fig. 5*a*), providing evidence that the solutes affect the conformation of the enzyme rather than the actual reaction between Nbf-Cl and exposed thiol groups.

We also noted that the phosphate anion, a structure stabilizer, decreases the rate of Nbf-Cl reaction with the enzyme and can offset the influence of urea (potassium phosphate as a buffer or the dibasic form added to other buffers; Yancey, 1978).

Protein-osmoregulatory-solute interactions

Mechanisms. The results of these and a preliminary study (Yancey, 1978) present strong evidence that these methylamine compounds and amino acids, used by certain marine animals for osmoregulation, will stabilize many inter- and intra-macromolecular interactions involving non-covalent bonds. Unfortunately, as in the case of neutral salt (Hofmeister series) and urea effects on macromolecules, this ability cannot be fully explained at present. The problem in determining mechanisms for many protein–solute interactions apparently lies in our poor understanding of the structure of water, particularly in the solvent 'shell' surrounding macromolecules in solution. For example, the Hofmeister series of ions may arise from a non-ranked binding of ions to protein amide dipoles modulated by nearby water-structuring groups (von Hippel *et al.*, 1973; Hamabata & von Hippel, 1973). And, although urea can form hydrogen bonds that may allow it to bind to proteins (e.g. Hermans, 1966), it can also destabilize hydrophobic interactions in proteins (Finch *et al.*, 1974), probably in an indirect manner by disrupting water structure (Finer *et al.*, 1972). However, these speculated interactions between solutes and the water around macromolecules are not adequately explained at present. Thus many of the effects of the methylamine and other urea antagonists on proteins, like the effects of urea and neutral salts, may not be understood until the organization and solute perturbations of water structure are better known.

Evolutionary implications. In organisms with high internal osmolalities the necessity for maintaining macromolecular structural and functional characteristics in the face of high solute concentrations is apparent. Three distinct evolutionary mechanisms have now been found for preserving macromolecular properties under such conditions. At one extreme, major changes in amino acid sequences of many proteins may occur, resulting in proteins with a tolerance of, or, in certain cases, a requirement for, high solute concentrations. The most dramatic changes in overall protein amino acid compositions related to intracellular osmotic content are found among the extremely halophilic bacteria, which have high internal concentrations of inorganic salts (Lanyi, 1974). But, even in certain proteins of marine elasmobranchs, amino acid substitutions related to urea tolerance may be important (Yancey & Somero, 1978). These latter changes appear to characterize urea-sensitive systems in which trimethylamine *N*-oxide has little stabilizing effect (Yancey, 1978).

However, there are alternatives to this mechanism. Rather than proteins evolving to fit the cellular solute environment, the solute composition of the cell may evolve to accommodate protein function as well as to maintain osmotic balance. As suggested in the introduction, this would involve the selection of compounds based on widespread, non-specific protein–solute interactions. There is evidence of one strategy of this kind, involving the accumulation of high concentrations of non-perturbing solutes, those that generally do not disturb protein function even at high solute concentrations. This apparently has happened in the case of unicellular algae, which accumulate the non-perturbing solute glycerol (Brown & Simpson, 1972; Borowitzka & Brown, 1974), and in the case of marine invertebrates, which accumulate several non-perturbing free amino acids (Bowlus & Somero, 1979). This type of adaptive pattern has been termed the use of 'compatible' solutes (Brown, 1976).

In the present study we have further described another strategy of cellular solute evolution. Marine elasmobranchs, holocephalans and the coelacanth appear to use solutes that individually perturb proteins but that together have negligible influences on a variety of protein systems as long as these counteracting solutes are present in certain ratios. Urea on the one hand, and certain methylamine compounds and free amino acids on the other, counteract each other at an approx. 2:1 molar concentration ratio (in most cases) as regards the kinetic properties of many enzymes (Yancey, 1978), protein thermal stability (Figs. 1 and 2), the kinetics of protein folding (Fig. 3), and the native conformation of an enzyme (Figs. 4 and 5). Although many of these effects were discovered with mammalian enzymes (used in order to compare results more directly with previous solute studies), our experiments with lactate dehydrogenases (Fig. 3) and on the kinetics of several enzymes from elasmobranchs, mammals and other vertebrates (Yancey, 1978) demonstrate that the counteracting influences of these solutes are qualitatively independent of the species source of protein. Thus the counterbalancing effects of these osmoregulatory compounds may occur with a wide variety of proteins in these marine fishes *in vivo*; the maintenance of an approx. 2:1 ratio of urea to the urea-antagonistic solutes intracellularly may be necessary to the maintenance of proper functional and structural properties of many proteins [e.g. the folding of nascent polypeptide chains as in Fig. 3, and optimal functional confor-

mations of enzymes (as suggested by the data in Figs. 4 and 5)].

The use of counteracting solutes for osmoregulation may occur in other animals as well. For example, in some marine invertebrates trimethylamine *N*-oxide is found at concentrations up to 0.1 M. It has been suggested that it may serve to offset the destabilizing effects of free arginine, a urea-like perturbant found in substantial concentrations in these animals (Bowlus & Somero, 1979). Trimethylamine *N*-oxide has also been found in many other animals (including mammals) at lower concentrations; its metabolic functions in such cases are not fully known. Whether its stabilizing abilities are important at low concentrations or in regions of locally high concentrations is also not known.

Conclusions

Our experiments and those of other workers (discussed above) establish that general interactions among solutes, proteins and water can, through natural selection, determine in part the solute compositions of cells. Indeed, evolutionary changes in intracellular solute systems, as well as changes in protein amino acid sequences, can play an important role in establishing the properties of proteins *in vivo*. At present, evidence for these conclusions has been based only on the biochemistry of organisms with very high intracellular concentrations of certain solutes. However, it may be that such general interactions among proteins and solutes in aqueous solution are important over evolutionary time in all organisms, especially in subcellular microenvironments having high concentrations of any type of solute. Thus the physiological significance of the effects of biological solutes on proteins warrants further investigation.

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