baric shoulder pressures seem to be fairly reproducible in the region of 46 kb. Similarly, the isothermal pressures cluster about 52 kb, in agreement with Stephens and Stromberg. Probably the lower value is closer to the true equilibrium pressure, since the dysprosium is presumably better equilibrated.

The 15- and 20-kb isobars were carefully measured in the temperature region above 77°K, but the Curie transition was not found. This confirms Swenson's report (*9*) that the Curie temperature decreases with pressure.

Two new properties of dysprosium may now be calculated by means of the Clausius-Clapeyron type equations for second-order transitions (*15*). At the Néel point

$$\frac{dT}{dP} = \frac{TV\,\Delta\alpha}{\Delta C_p} \quad , \quad \frac{dT}{dP} = \frac{\Delta\beta}{\Delta\alpha}$$

where $\Delta\alpha$, $\Delta\beta$, and ΔC_p are the changes in the coefficient of linear expansion, compressibility, and heat capacity at constant pressure, respectively. The values for the 1-atm Néel point are -0.62 deg/kbar and 8.5 cal/deg mole^{-1} (*16*) for dT_N/dP and ΔC_p, respectively. Values of $\Delta\alpha$ and $\Delta\beta$ are calculated to be -64×10^{-6} $(°C)^{-1}$ and 4.0×10^{-14} cm^2/dyne, respectively. By way of comparison, α is 8.5×10^{-6} $(°C)^{-1}$ near the Néel point (*17*) and β is 2.44×10^{-12} cm^2/dyne at room temperature (*18*). The same magnetic forces that cause the lattice parameter c to increase below the Néel temperature are probably related to the very large change in the coefficient of linear expansion.

The resistivity-pressure-temperature curves found in this investigation are rather complex. Above about 75 kb, all of the results appear to be normal in that there is a decrease in resistance with an increase in pressure. Below this pressure there are a number of unusual features in the resistivity behavior. Many different hypotheses can be presented to explain the curious behavior that has been observed. However, on the basis of the available data, no conclusions can be drawn that would appear to be reasonably sound to us.

The nature of the change that occurs at about 50 kb on an isothermal compression is also a complex question. This point is discussed in detail by Jamieson in an accompanying paper (*19*). Professor Jamieson has kept us informed of the progress of his x-ray diffraction work as it progressed. The magnetic nature of dysprosium at the higher pressures is unknown. The one conclusion that seems to be inevitable from both of these investigations is that there is a magnetic change above the cusp. There is also an uncertainty concerning the exact value of the pressure at which the transformation occurs, since there is a discrepancy between the values obtained from the isothermal and isobaric work. Fortunately, this discrepancy is only of about 5 kb.

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22 June 1964

Neutral Salts: The Generality of Their Effects on the Stability of Macromolecular Conformations

Abstract. *The effects of various neutral salts on the temperature of the thermally-induced denaturation of the globular protein ribonuclease are described and compared with the effects of these salts on helix-coil transition temperatures in other macromolecules. These agents affect the stability of the native form of macromolecules as diverse as ribonuclease, collagen, DNA, and myosin in very similar ways; salts such as KSCN and $CaCl_2$ serve as very potent general structural destabilizers or denaturants, while salts such as $(NH_4)_2SO_4$ and K_2HPO_4 strongly stabilize the native conformation. The effectiveness of the neutral salts as ribonuclease destabilizers is compared with that of urea and the guanidinium salts.*

For many years, intramolecular (primarily peptide) hydrogen bonds were considered to be the main noncovalent source of structural stability in native proteins and nucleic acids. Thus the capacity of denaturing agents, such as urea and guanidinium chloride, to disrupt secondary-tertiary structure has generally been attributed to their effectiveness as competitive hydrogen-bond formers. Recently evidence has been accumulating which suggests that nonpolar interactions (hydrophobic bonds), rather than hydrogen bonds, play the dominant role in the maintenance of the native conformation in aqueous solution (*1*). Such views provide a rational explanation of the denaturing activity of largely nonpolar compounds such as sodium dodecyl sulfate. However, the neutral salts, though generally devoid of either obvious hydrogen bonding or hydrocarbon-solubilization potential, can also markedly perturb secondary-tertiary structure. It is the purpose of this report to demonstrate the remarkable generality of the effects of neutral salts on the stability of macromolecular conformations.

Macroscopically, the striking conformational effects of neutral salts were early recognized in their influence on the shrinkage temperature of collagen fibers (*2*) and the melting temperature of gelatin gels (*3*). In comparative studies with a common cation or anion it was found that the ions could be ranked in order of increasing effectiveness in lowering the

shrinkage or melting temperature (temperature decrease per mole of ion added), essentially as listed in the top portion of Fig. 1. Subsequently, it was shown by Flory and Garrett (*4*) that these melting or shrinkage phenomena are simply macroscopic manifestations of the reversible helix to coil transition in which the rigid collagen macromolecule, with its three chains each wound into a slightly deformed, left-handed, poly-L-proline II helix, collapses into an essentially random-coil form (*5*). In an optical rotatory study of the effects of ions on the melting temperature (T_m, defined as the temperature of the mid-point of the transition) of collagen and cold gelatin in dilute solution (*6, 7*) it was demonstrated that the series listed in Fig. 1 also describe the order of effectiveness of the various ions in displacing the T_m of the molecular helix-coil transition.

At about the same time it was shown by Hamaguchi and Geiduschek (*8*) that a similar series applies to the effect of the various ions, at high concentrations, on the temperature of the helix-coil transition in DNA (Fig. 1). The similarity of these effects on two such apparently unlike macromolecules as collagen and DNA led us to examine the effects of ions on the T_m of an order-disorder transition in a globular protein. To this end we have studied the well-known thermally induced cooperative unfolding of ribonuclease which occurs at about 60°C in dilute salt solutions near neutral *p*H (*9*). It had previously been shown that the T_m for this transition is lowered in the presence of increasing quantities of LiBr (*10*).

Optical rotary measurements were carried out in a temperature-controlled one-decimeter cell at 366 mμ, with a Rudolph Model 80 spectropolarimeter equipped with a rocking polarizer and a photoelectric read-out device. Samples (5 mg/ml) of bovine pancreatic ribonuclease (*11*) were heated progressively from room temperature to about 70°C, measurements being made at intervals of about 5°C after a 10-minute wait for equilibrium at each temperature. Melting curves ($[\alpha]_{366}$ plotted against temperature) were obtained at various concentrations of each salt tested. Some of the results are plotted as T_m against the molarity of added salt in Fig. 2. These and other experiments (*12*) led to ion-effectiveness series for ribonuclease (Fig. 1)

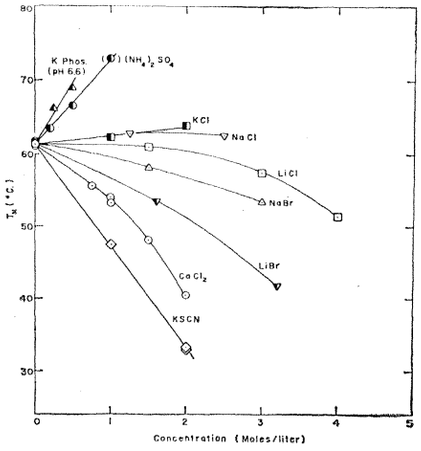

Fig. 2. Melting temperature for the ribonuclease transition as a function of concentration of various added salts. The curves for potassium phosphate and $(NH_4)_2SO_4$ were not extended further because higher concentrations of these electrolytes precipitated (salted-out) the ribonuclease from solution.

which are very similar to those obtained with collagen-gelatin and DNA. Here also ions such as $H_2PO_4^-$ ($HPO_4^=$) and $SO_4^=$ increase T_m (stabilize the native conformation), K^+, Na^+ and Cl^- have little effect, and Li^+, Ca^{++}, Br^-, and SCN^- markedly destabilize the native conformation. Both anions and cations are effective, and their effects are roughly additive. For example, in Fig. 2 we may see that at a given molarity the effect on T_m of LiBr plus NaCl is approximately equal to that of LiCl plus NaBr.

By comparison with some of the neutral salts, the classical denaturing agents, urea (*13*) and guanidinium chloride, are only moderately potent. As a melting temperature depressor (destabilizer) for ribonuclease, urea falls betweeen NaBr and LiBr, and guanidinium chloride between $CaCl_2$ and KSCN (compare Figs. 2 and 3). We have also found, as might have been expected from Greenstein's early work on the exposure of sulfhydryl groups attendant on the denaturation of ovalbumin (*14*), that the guanidinium salts differ markedly in their effects on the T_m of the ribonuclease transition (Fig. 3). Thus guanidinium sulfate actually increases T_m, the stabilizing effect of the sulfate anion more than offsetting the destabilizing effect of the guanidinium cation. At the other extreme, the destabilizing effect of the thiocyanate anion combines with that of the guanidinium cation to make guanidinium thiocyanate a much more effective denaturing agent than the traditional guanidinium chloride.

	← Helix	Coil →
	← (Salting-out)	(Salting-in) →
Collagen – Gelatin	$SO_4^= < CH_3COO^- < Cl^- < Br^- < NO_3^- < ClO_4^- < I^- < CNS^-$	$(CH_3)_4N^+ < NH_4^+ < Rb^+, K^+ Na^+, Cs^+ < Li^+ < Mg^{++} < Ca^{++} < Ba^{++}$
		$(CH_3)_4N^+ < (C_2H_5)_4N^+ << (C_3H_7)_4N^+, (C_4H_9)_4N^+$
Ribonuclease	$SO_4^= < CH_3COO^- < Cl^- < Br^- < ClO_4^- < CNS^-$	$(CH_3)_4N^+, NH_4^+, K^+, Na^+ < Li^+ < Ca^{++}$
		$(CH_3)_4N^+ < (C_2H_5)_4N^+ < (C_3H_7)_4N^+ < (C_4H_9)_4N^+$
DNA	$Cl^-, Br^- < CH_3COO^- < I^- < ClO_4^- < CNS^-$	$(CH_3)_4N^+ < K^+ < Na^+ < Li^+$
Benzoic Acid	$Cl^- < Br^- < I^- < NO_3^- < ClO_4^- < CNS^-$	$Li^+ < Na^+ < K^+ < NH_4^+ << (CH_3)_4N^+ < (C_2H_5)_4N^+$

Fig. 1. Relative effectiveness of various ions in stabilizing or destabilizing the native form of collagen, ribonuclease, and DNA, and in salting benzoic acid into or out of aqueous solution. Collagen-gelatin data (*3, 6, 7*); ribonuclease data, this study and (*12*); DNA data (*8*); benzoic acid data (*18*).

Once one adopts this general view of the effect of the various ions on macromolecular stability, other illustrative examples are readily culled from the literature. For example from the work of Tonomura *et al.* (*15*) on the fibrous muscle protein myosin, we may derive the following series:

$$KCl < LiCl < LiBr < KI < KSCN$$

Here the salts are ranked in order of increasing effectiveness in reducing the helix content of the myosin molecule.

To provide a quantitative comparison of some of these data, ΔT_m, the increase or decrease in T_m from its value at zero concentration of additive, is plotted against the molarity of added salt in Fig. 4. Clearly the effect of the ions shown on the stability of conformations as diverse as the DNA helix, the collagen-type helix, the α-helix (myosin), and a folded globular protein (ribonuclease) are very similar (*16*).

These equilibrium effects on conformational stability also have their kinetic counterparts. The ion series of Fig. 1 (reading from left to right) also have been shown (*7*) to apply to the effectiveness of the various ions in decreasing the rate of formation of the collagen-type helix after quick-cooling of warm random-coil gelatin to a fixed temperature below T_m. As one might expect from the equilibrium results, $SO_4^=$ and $(CH_3)_4N^+$ actually increase the rate of helix formation above that observed in dilute solution. Simpson and Kauzmann (*17*), in a study of the kinetics of denaturation of ovalbumin by urea, also showed that various

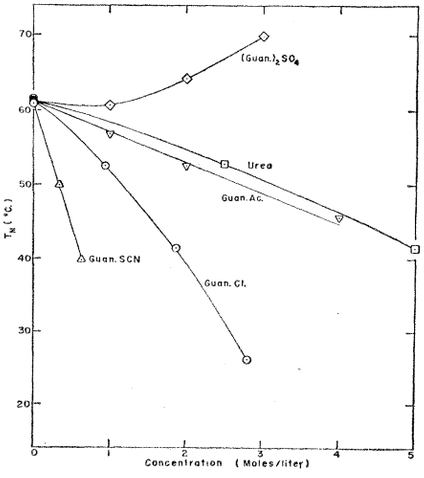

Fig. 3. Melting temperature for the ribonuclease transition as a function of concentration of urea and various guanidinium salts.

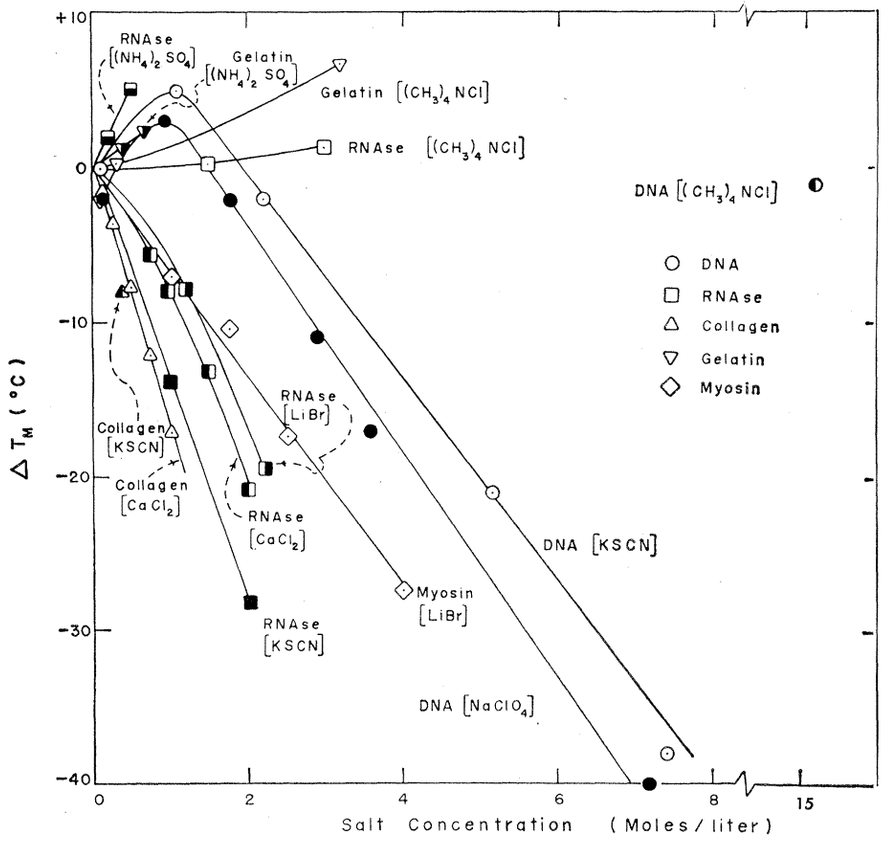

Fig. 4. Change (increase or decrease) in melting temperature for helix-coil transitions in various macromolecules as a function of concentration of added salts. Ribonuclease data, this study, and (*12*); collagen-gelatin data (*6, 7*); DNA data (*8*); myosin data (*15*).

salts altered the rate of denaturation in the following order of increasing effectiveness: Na_2SO_4, NaH_2PO_4, $NaCH_3COO$, NaCl, $NaNO_3$, $BaCl_2$, KI, $MgCl_2$, KSCN, and $CaCl_2$. In this series salts to the left of NaCl decreased the rate of denaturation, while those to the right of NaCl increased the rate.

The mechanism (or mechanisms) by which these neutral salts alter the stability of macromolecular conformations cannot be unequivocally defined at present. However, the available data do permit us to rule out certain possibilities. (i) The effects are clearly not electrostatic or charge-shielding in nature, since identical concentrations (activities) of salts of the same valence type have such different effects. (ii) The magnitude of the stabilization or destabilization shows no correlation with the effect of the various salts on the activity of water (*6, 8*). (iii) The effects are qualitatively and even semiquantitatively (Fig. 4) the same in direction and magnitude for all the macromolecules considered, indicating that specific chemical or conformational properties of the individual macromolecules are not critical.

Certain additional observations also bear on the generality of these neutral salt effects and thus on possible mechanisms. For example, the same ion series (Fig. 1) generally apply to the relative effectiveness of these electrolytes in salting-in (increasing effectiveness to the right) and salting-out (increasing effectiveness to the left) proteins. Furthermore, similar anion series are observed for simpler processes such as the salting-out of benzoic acid and other small molecules from water (*18*), though it should be noted that the cation series is reversed in the benzoic acid case (Fig. 1). In view of these correlations, and since the ions in Fig. 1 are arranged essentially in the Hofmeister series which applies to so many ionic phenomena, it would seem probable that the effects on macromolecular conformations are a consequence of very general effects of the various ions on the structure of the solvent, which in turn modify solvent-macromolecule interactions involved in the stabilization of the native structures (*6–8*). However, despite the generality of these effects, it should be recognized explicitly that specific interactions between ions and macromolecules can and do occur, and that

such interactions may alter the stability of ordered conformations by special and quite different means.

In conclusion, and quite apart from considerations of mechanism, we wish to emphasize the empirical fact that certain neutral salts are very effective general macromolecular denaturants, and that others are rather potent general stabilizers of macromolecular conformations. We may note that highly concentrated solutions of salts of the latter type (K_2HPO_4, $(NH_4)_2SO_4$ and so forth) are often used to salt-out native proteins, and as the mother liquor for x-ray diffraction studies of proteins assumed to be native in the crystalline state.

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Recent High Relative Sea Level Stand near Recife, Brazil

Abstract. *Radiocarbon dates for Vermetidae limestone from the edge of the Brazilian Shield at Cape San Agostinho, Brazil, indicate sea level stands of up to 2.60 meters above the present position, 3660, 2790, and 1190 years ago. Accurate determination of relative sea level is possible because of the well-defined habitat of the fossils dated.*

Shepard (*1*) has listed three current trends of thought about the rise of sea level during the last few thousand years: (i) during the past 6000 years, sea level has fluctuated between 1.5 m below and 3.0 m above its present position; (ii) sea level has been constant since it reached its present position some 3000 to 5000 years ago; and (iii) sea level has continued to rise slowly up to the present day. Shepard presents new information to support the last viewpoint. However, his graphs show considerable scatter of dates around the mean curve. The scatter exceeds several thousands of years for each position of sea level, or some 3 to 6 m for each point in time. Obviously, absolute sea level must have occupied a single position at each specific time. Consequently, one of the following alternatives must apply to each date to explain this scatter, provided that it is truly related to ancient sea level and not to an artifact (for example, a kitchen midden). (i) The date is in error because of faulty measurement or contamination with lighter or heavier carbon. (ii) The inferred relation to ancient sea level is incorrect. (iii) The area is unstable and either uplift or subsidence has occurred. (iv) The rise of sea level has indeed shown many fluctuations, some of them above present level, through all or many of the points given on the graphs (*2*).

Thus, there appears to be an urgent need for additional data that satisfy stringent requirements of purity of the sample, well-defined relation to ancient sea level, and stability of the region. In this report we present four dates obtained from measurements of biogenous limestone that encrusts parts of a small promontory of granite on Cape San Agostinho near the village of Gaibú, south of Recife, Brazil. The sample location is 34°56.0′ west longitude and 8°20.2′ south latitude (*3*). The area forms part of the eastern margin of the Brazilian Shield.

At Gaibú, the mean range for spring tides is 2.3 m with a maximum of 2.7 m, and the coast is exposed to a considerable swell. The following biological zonation occurs (Fig. 1): a *Littorina* zone in the upper part of the tidal range from 2.00 to 2.30 m above mean low water, corresponding to the "étage supralittoral" of Peres and Molinier (*4*); a *Chthamalus* zone in the middle upper part of the tidal zone from 1.40 to 2.00 m above mean low water, the upper part of the "étage médiolittoral" (the precise level of this zone varies, depending on exposure to the surf); a *Tetraclita* zone from 0.20 to 1.3 m above mean low water, corresponding to the lower part of the "étage médiolittoral"; a Vermetidae zone of thick concretions of encrusting gastropods, algae, and Foraminifera quite similar to those from the upper part of the "étage infralittoral" of the Mediterranean described by Molinier (*5*) [the dominant invertebrate in this zone, which is limited to a range from 0.30 to 0.80 m above mean low water, is *Petaloconchus* (*Macrophragma*), probably a variant of *P. varians* d'Orbigny (*6*)]; a *Sargassum* zone, infralittoral, extending below low water and including scarce coral heads.

Fossil Vermetidae limestone accretions attached under overhanging solid rock surfaces, or filling joints and cementing blocks, occur at various

Table 1. Radiocarbon dates of four samples of limestone from Gaibú, Brazil. (M.L.W., mean low water.)

Sample No.	Height above M.L.W. (m)	Height above top of living vermetidae zone (m)	Age (years)	Description
A-16	3.00	2.20	2790±150	Biogenous limestone with Vermetidae, Balanidae, and encrusting Foraminifera
A-17	2.40	1.60	1190±130	Biogenous limestone, mainly Vermetidae
A-21	2.20	1.40	1750±170	Biogenous limestone with Vermetidae and Bryozoa
A-22	3.40	2.60	3660±170	Biogenous limestone with Vermetidae