Ion-Induced Water-Proton Chemical Shifts and the Conformational Stability of Macromolecules*

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ABSTRACT: Water-proton chemical shift measurements have been made as a function of concentration and temperature for a number of aqueous electrolyte solutions capable of destabilizing the folded (native) conformations of macromolecules, and attempts have been made to correlate these chemical shifts with the effectiveness of the salts in lowering the transition temperature (T_m) of ribonuclease and other macromolecules. The procedures used are comparable with those previously employed by Gordon *et al.* (1965) with DNA in aqueous sodium perchlorate solutions. As found for the DNA–perchlorate system, ribonuclease in variously concentrated sodium perchlorate solutions "melts" at a constant water-proton chemical shift (provided that the contribution of the sodium ion is removed). This result shows that macromolecules in general can be expected to undergo thermal denaturation at particular constant water "structure temperatures," defined as a constant value of the corrected water-proton chemical shift, whether this value is attained by heating the solution, or isothermally by the addition of sodium perchlorate. In attempting to extend this correlation to other destabilizing ionic solutes, we have found that the simple quantitative relationship observed with perchlorate is *not* maintained, though suggestive trends continue to be observed. Various empirical corrections to the chemical shift values do not restore the correlation. We conclude that the water-proton chemical shift, like other parameters measured on two-component water–salt systems, does not provide a general and unambiguous *quantitative* measure of those aspects of water structure involved in determining the stability of macromolecular conformations.

The structure of liquid water, both alone and in the presence of various additive electrolytes and nonelectrolytes, has been of considerable interest to physical chemists for many years. Recently this problem has also attracted the attention of physical biochemists, since it has become increasingly clear that the ordered (native) conformation of biological macromolecules is largely determined and stabilized by "hydrophobic" bonds, in which the structure of water and its modification in the vicinity of nonpolar groups play a crucial role (Kauzmann, 1959). The addition of a variety of charged and uncharged low molecular weight solutes to the aqueous surroundings of proteins and nucleic acids alters the stabilities of the folded structures of these macromolecules with respect to a thermally induced conversion into a more solvated (unfolded) form, in a way which is quite independent of the details of macromolecular composition and structure (von Hippel and Wong, 1964; von Hippel and Schleich, 1969a,b). Model compound studies have suggested that these additive-induced changes in conformational stability are primarily a consequence of alterations in the free energy of transfer of various groups from the nonaqueous interior of the macromolecule into the solvent (Nozaki and Tanford, 1963; Robinson and Jencks, 1965a,b; Robinson and Grant, 1966; Schrier and Schrier, 1967). Solvent perturbants can influence this free energy of transfer either directly by binding to the newly exposed groups, or indirectly by modifying the structure of the solvent surrounding these groups, or most likely by a combination of both mechanisms. In order to assess the contributions of solute-induced changes in water structure to macromolecular stability, it is necessary to develop measures of water structure modification which can be compared directly with macromolecular effects. Many physical measures of water structure exist (for recent reviews, see Kavanau, 1964; Samoilov, 1965; von Hippel and Schleich, 1969a; Eisenberg and Kauzmann, 1969), but most of these measures appear to be sensitive to specific features of water-perturbant interaction in addition to those aspects presumably sensed by the macromolecule.

One approach which has given promise of providing an experimental test for correlations between ion-induced water structure modifications and macromolecular stability is a study of the temperature dependence of the water-proton nuclear magnetic resonance chemical shift in variously concentrated electrolyte solutions. The effects of different electrolytes on the water-proton chemical shift as a function of salt concentration have been studied isothermally (usually at 25°) by a number of workers (Shoolery and Alder, 1955; Hertz and Spalthoff, 1959; Fabricand and Goldberg, 1961; Hindman, 1962; Bergqvist and Forslind, 1962; Glick *et al.*, 1966). The temperature dependence of this chemical shift in the presence of some ionic solutes has been examined by Hertz and Spalthoff (1959), while Gordon *et al.* (1965) have made a detailed nuclear magnetic resonance study of the aqueous sodium perchlorate system as a function of both temperature and solute concentration. The perchlorate anion is an effective macromolecular denaturant, *i.e.*, the temperature at which a cooperative unfolding of the macro

* From the Department of Chemistry and the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received September 30, 1969.* This investigation was supported in part by Research Grants AM-03412, AM-12215, GM-15792, and a Research Career Program award (GM-K3-5479) from the United States Public Health Service (to P. H. von Hippel), and a Helen Hay Whitney Foundation postdoctoral fellowship (to Thomas Schleich).

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molecule takes place (T_m) is progressively lowered by increasing concentrations of perchlorate, and after correcting the observed water-proton chemical shift for sodium ion and bulk susceptibility effects, Gordon *et al.* (1965) were able to show that in variously concentrated perchlorate solutions native calf thymus DNA is denatured at a constant value of the water-proton chemical shift. This result was interpreted by these workers to imply that DNA melts in sodium perchlorate solutions at a constant liquid "structure temperature" (Bernal and Fowler, 1933) defined by the magnitude of the chemical shift, whether this structure temperature is attained by simple heating, or isothermally by the addition of perchlorate anion. To take the argument one step further, this suggests that perchlorate affects the stability of DNA entirely indirectly, operating as a water structure disorganizer in the same sense as water structure is disorganized by increasing temperature. Furthermore, these results suggest that the appropriately corrected magnitude of the ion-induced chemical shift of the water-proton resonance might serve as a direct measure of the effectiveness of various solvent additives as macromolecular destabilizers.

With these implications in view, we undertook the present study to see how far these concepts could be carried, both with respect to other macromolecular transitions and to structure-perturbing electrolytes other than sodium perchlorate. To this end we have studied in detail the concentration and temperature dependence of the water-proton chemical shift of a number of electrolyte solutions of varying potency as macromolecular destabilizers, and have attempted to correlate the measured shifts with the thermal stability of ribonuclease and other macromolecules in these solutions. The results of this study show that ribonuclease, like DNA, melts in various concentrations of aqueous sodium perchlorate at a constant value of the (sodium corrected) water-proton chemical shift. However, while suggestive trends are observed, we find that this simple quantitative correlation between chemical shift and macromolecular melting temperatures does not hold in general for other destabilizing salts.

Materials and Methods

The salts NaCl, NaI, NaSCN, LiCl, LiBr, and $CaCl_2$ were obtained as certified reagents from the Fisher Scientific Co. Reagent grade LiI (trihydrate) and RbCl were purchased from Alpha Inorganics, and reagent grade $NaClO_4$ (anhydrous) from G. F. Smith Co. Tetramethylammonium chloride was Eastman White Label grade. All salts were used without further purification. HMDS[1] was purchased from K and K Laboratories. Ribonuclease (lot 126B-2070) was obtained from Sigma, and was type II-B, activity 60 units/mg, protease free and essentially salt free, containing 60–80% Hirs fraction A.

Salt concentrations were determined by argentometric titration using sodium fluoresceinate as an adsorption indicator (Kolthoff and Sandell, 1952), though for some salts direct weighing was also used. The choice of salt concentration units was a problem, since in previous nuclear magnetic resonance work on ion-induced proton-chemical shifts the *molal* scale has invariably been used, while the *molar* scale has been employed for studies of the effects of ions on macromolecular melting temperatures. In this paper *both* scales are used, the molal scale being primarily used for the nuclear magnetic resonance data and the results converted into molar units for comparison with T_m data obtained in other studies. Conversion from one set of units into the other was made using concentrations and densities determined by weighing 5.0-ml volumetric flasks containing the appropriate solutions.

Water-proton chemical shifts were measured relative to an external standard of HMDS in precision-bore concentric nuclear magnetic resonance tubes (Wilmad 5 mm o.d.)[2] using a Varian DA-60-IL spectrometer system outfitted with the usual accessories for high-resolution proton spectroscopy at 60 MHz. The sample temperature was maintained by the use of a Varian V-6040 gas-flow temperature controller, which was initially calibrated by placing a thermistor connected to a Yellow Springs 42SC telethermometer into a water-filled nuclear magnetic resonance tube mounted in the probe. This calibration was checked against the position of the temperature-sensitive water resonance line periodically throughout the course of these experiments. Temperature measurements were found to be reliable to $\pm 1°$.

All chemical shift values reported here represent the average of at least five separate determinations, and all have been corrected for bulk susceptibility differences by measuring the splitting of the water proton signal in a nonspinning concentric sample tube. Bulk susceptibility corrected chemical shifts are expressed in parts per million relative to ethane using the following formula (Malinowski *et al.*, 1966)

$$\delta_{\text{cor.,sol}} = \delta^{\text{HMDS}}_{\text{obsd,sol}} - \delta^{\text{HMDS}}_{\text{obsd,H}_2\text{O}} + (r^2/6b^2)(\Delta_{\text{sol}} - \Delta_{\text{H}_2\text{O}}) + \delta_{\text{corr,H}_2\text{O}}$$

in which the δ terms refer to the chemical shift values in parts per million, $r^2/6b^2 = 0.1710$ for the nuclear magnetic resonance tubes employed, and the Δ terms are the splittings in parts per million of the signal obtained from nonspinning sample tubes; $\delta_{\text{corr,H}_2\text{O}}$ values (relative to ethane) were taken from Malinowski *et al.* (1966). The uncertainty in the chemical shift measurements is ± 0.01 ppm. It should be noted that by the convention adopted in this paper negative parts per million values reflect a chemical shift downfield from the reference material. The ionic solutions used in the nuclear magnetic resonance investigations did not contain ribonuclease, since it has been shown that macromolecules at concentrations of 1–10 mg/ml do not affect the position of the water-proton resonance line (Gordon *et al.*, 1965).

The thermal transition temperature (T_m) of ribonuclease was determined by following the normalization of the buried tyrosines at 287 mμ as a function of temperature using a temperature programmed Gilford Model 2000 recording spectrophotometer. Ribonuclease was dissolved to a final concentration of 0.5 mg/ml in pH 6.8, 0.01 M cacodylate

[1] Abbreviation used which is not listed in *Biochemistry* 5, 1445 (1966), is: HMDS, hexamethyldisiloxane.

[2] These tubes were of a modified design to fit into an HA-100 type probe and spinner assembly (tuned to 60 MHz). The inner tube was the standard supplied length while the outer tube was increased in length so that when assembled 2 mm of the inner tube projected beyond the end. The entire tube assembly was sealed with a Wilmad pressure cap.

TABLE I: Selected Nuclear Magnetic Resonance Water-Proton Chemical Shift Data for Ionic Solutions.

Salt	Molarity	Molality	$-\delta_{25°}$ (ppm)	$-\delta_{corr,\,25°}$ (ppm)	$d\delta/dT$ (ppm/°C)	r^a
Water			5.08	4.13	0.0093	0.998
NaCl	1.90	1.99	5.01	3.99	0.0080	1.000
NaCl	3.67	3.99	4.94	3.85	0.0065	0.999
NaCl	5.16	5.81	4.92	3.80	0.0060	0.995
NaI	1.85	1.99	4.96	3.88	0.0080	1.000
NaI	3.40	3.91	4.86	3.68	0.0072	0.999
NaI	4.98	6.23	4.78	3.50	0.0060	0.999
LiCl	1.83	1.91	5.10	4.10	0.0077	0.999
LiCl	3.68	4.00	5.13	4.09	0.0070	1.000
LiCl	5.06	5.65	5.14	4.07	0.0062	0.999
LiI	1.74	1.87	5.11	4.06	0.0105	0.994
LiI	2.90	3.26	5.07	3.96	0.0092	0.994
LiI	3.78	4.42	5.04	3.88	0.0085	0.995
RbCl	1.83	1.97	5.04	4.00	0.0080	0.999
RbCl	3.39	3.87	4.99	3.88	0.0067	0.999
RbCl	4.64	5.55	4.97	3.80	0.0060	1.000
$NaClO_4$	1.84	2.00	4.85	3.88	0.0083	0.999
$NaClO_4$	3.47	4.00	4.68	3.69	0.0078	0.998
$NaClO_4$	4.68	6.00	4.55	3.56	0.0075	0.999
$(CH_3)_4NCl$	1.63	1.99	5.04	4.08	0.0085	1.000
$(CH_3)_4NCl$	2.73	3.89	5.02	4.05	0.0080	0.997
$(CH_3)_4NCl$	3.65	6.03	6.03	4.05	0.0077	0.999

[a] Regression coefficient for linear least-squares fit of five points.

buffer (made up from cacodylic acid by titration with NaOH) which contained the salt under investigation. No corrections to the absorbance were made for thermal expansion of the solvent. T_m was defined in the usual way as the temperature of the midpoint of the sigmoid change of absorbance with temperature.

Results

At the time we initiated this study, many workers had determined the salt concentration dependence of the water-proton chemical shift in a variety of aqueous electrolyte solutions. However measurements of the temperature dependence of these chemical shifts for most of the salts in which we were interested were not available. Thus we undertook to make such measurements. After the completion of our experimental work, a paper appeared by Creekmore and Reilley (1969), in which determinations of the water-proton chemical shift as a function of temperature were reported for a number of the electrolyte systems which we had measured independently. In general our results are very close to those of Creekmore and Reilley, and thus we report them here only in sufficient detail to permit the reader to make comparisons, and to follow the use to which we put these data in attempting to establish correlations with macromolecular stability.

Some of the nuclear magnetic resonance data we obtained are summarized in Table I, in which we list the measured and bulk susceptibility corrected water-proton chemical shift values for a number of representative salt solutions at three different concentrations and 25°, as well as the slope ($d\delta/dT$) obtained from a plot of δ_{corr} *vs.* temperature (5–85°) for each solution, and r, the correlation coefficient for each least-squares slope. In general, our values of $\delta_{corr,\,25°}$ are in good accord with those of Hindman (1962), and our measurements of $d\delta/dT$ agreed well with those of Creekmore and Reilley (1969) when extrapolated to the same electrolyte concentrations. The correlation coefficients listed in Table I (all greater than 0.994) show that within the accuracy of the data the water-proton chemical shift for a given salt solution is a linear function of temperature. However Table I also shows that $d\delta/dT$ for a given salt depends on the salt concentration, generally decreasing with increasing concentration, though this trend is more pronounced for some salts (*e.g.*, NaCl, LiI) than for others (*e.g.*, $NaClO_4$, $(CH_3)_4NCl$). These aspects of the dependence of δ_{corr} on temperature and salt concentration are illustrated graphically for NaCl in Figure 1, and for LiCl in Figure 2.

Ribonuclease transition temperatures, obtained by following absorbance changes at 287 mμ (*e.g.*, see Hermans and Scheraga, 1961a,b) in the same solutions used in the chemical shift studies, are tabulated in Table II. These values are in good accord with those previously determined by polarimetric (von Hippel and Wong, 1965) and spectrophotometric (Schrier and Schrier, 1967) techniques. As previously shown (von Hippel and Wong, 1964, 1965) and as is also apparent from Table II, T_m depends on both the type and the concentration of salt added. Furthermore, anions and cations are indepen-

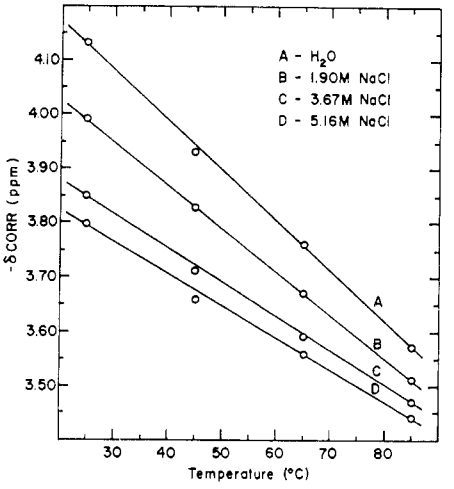

FIGURE 1: The variation with temperature of the water-proton chemical shift (corrected for bulk susceptibility) of variously concentrated NaCl solutions.

dently and additively effective in altering the transition temperature of ribonuclease, and for most salts, though not all, the dependence of T_m on salt molarity (or molality) is approximately linear (von Hippel and Wong, 1964, 1965; Talbot, 1968). In order of increasing effectiveness as macromolecular destabilizing agents (T_m depressants) the anions examined in this study may be ranked: $Cl^- < Br^- < ClO_4^- < SCN^-$. The cations follow the order: $Na^+ < Li^+ < Ca^{2+}$. (For much more extensive compilations and discussions of ion effects on the stability of macromolecules, see von Hippel and Schleich, 1969a). One other fact which is pertinent to what follows is that the molar effectiveness of a given destabilizing salt in perturbing T_m is essentially independent of the macromolecular type and of the absolute value of the transition temperature. Thus for most salts examined, the molar shift in T_m (ΔT_m/mole) is approximately the same (within $\sim\pm 20\%$) for calf thymus DNA ($T_m^\circ \simeq 90°$), pancreatic ribonuclease ($T_m^\circ \simeq 60°$), and (ichthyocol) collagen ($T_m^\circ \simeq 30°$). (T_m° is

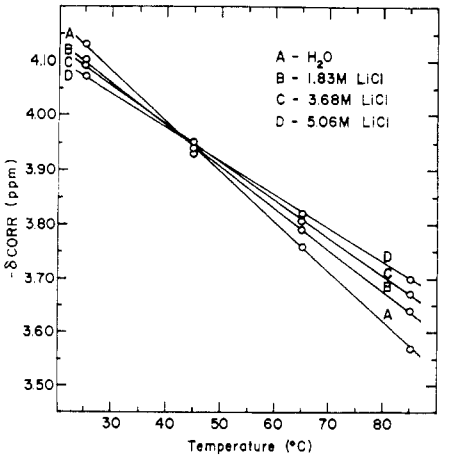

FIGURE 2: The variation with temperature of the water-proton chemical shift (corrected for bulk susceptibility) of variously concentrated LiCl solutions.

TABLE II: Ribonuclease Transition Temperatures in Various Ionic Solutions.

Solution (M)	T_m (°C)[a]
"0" (pH 6.8, 0.10 M cacodylate buffer)	62.3
NaCl (1.9)	64.5
NaCl (3.0)	65.7
NaCl (3.7)	66.5
NaBr (1.9)	57.2
NaBr (3.0)	54.2
NaBr (3.6)	51.7
NaBr (5.1)	37.0
LiCl (1.8)	60.1
LiCl (3.7)	54.0
LiCl (5.1)	35.1
LiBr (1.9)	50.1
LiBr (3.6)	29.2
NaSCN (0.9)	48.0
NaSCN (1.9)	36.9
NaSCN (2.8)	25.5
NaSCN (3.0)	21.2
$CaCl_2$ (0.9)	54.0
$CaCl_2$ (1.8)	44.0
$NaClO_4$ (0.5)	55.6
$NaClO_4$ (1.0)	51.0
$NaClO_4$ (1.5)	46.1
$NaClO_4$ (2.0)	42.2
$NaClO_4$ (2.5)	38.7
$NaClO_4$ (3.0)	34.5

[a] Average of three determinations.

defined as the melting temperature of the macromolecule in dilute neutral salt solution.)

Macromolecule transition temperatures are correlated with nuclear magnetic resonance water proton chemical shifts as follows (Gordon *et al.*, 1965): On plots of the bulk susceptibility corrected water-proton chemical shift as a function of temperature for a given salt at a number of concentrations (*e.g.*, Figures 1 and 2) we indicate on the line for each salt concentration the temperature at which the macromolecule melts in that solution. A line connecting these points then represents the relation between T_m and δ_{corr} for that macromolecule. If this line is straight and horizontal, we conclude that melting occurs at a constant value of δ_{corr}, or at a constant water structure temperature as defined by this parameter.

Figure 3 shows T_m data for ribonuclease in various concentrations of $NaClO_4$, superimposed on a series of plots of δ_{corr} *vs.* temperature for $NaClO_4$ solutions. Clearly δ_{corr} is not invariant with T_m here; rather a line of positive slope is found.

If we subtract the contribution of sodium ion to the total corrected chemical shift for sodium perchlorate solutions, an excellent horizontal T_m line results (Figure 4), just as observed previously with DNA (Gordon *et al.*, 1965). The rationale for making a sodium ion correction to the chemical shift is discussed below; the procedures used were identical with those employed by Gordon *et al.* (1965) and are based on ionic chemical shift values defined by Hindman (1962).

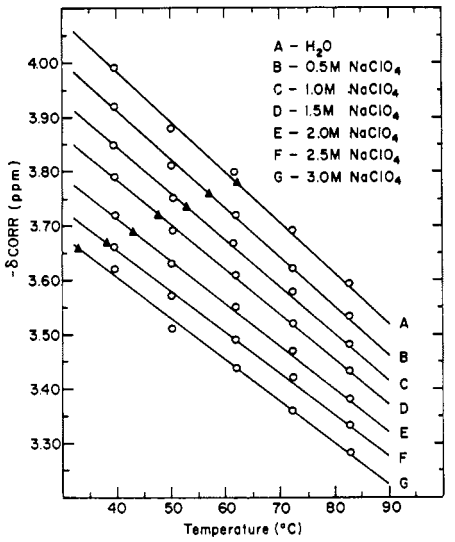

FIGURE 3: The variation of the sodium perchlorate induced water-proton chemical shift with temperature in variously concentrated solutions. Superimposed on the plot are the transition temperatures of ribonuclease in these same solutions (▲).

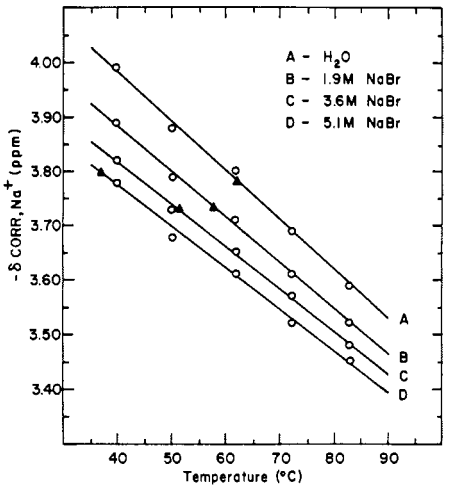

FIGURE 5: The variation of the sodium ion corrected sodium bromide induced chemical shift of water protons with temperature in variously concentrated sodium bromide solutions. Superimposed on the plot are the transition temperatures of ribonuclease in these same solutions (▲).

In other salt–macromolecule (ribonuclease) systems, the T_m points describe nonlinear or nonhorizontal lines even after the sodium ion correction of δ_{corr}. Figure 5 shows that a concave upward T_m curve is obtained for different concentrations of the moderately destabilizing Br^- anion. For various concentrations of the more effective destabilizer SCN^-, the T_m points on a plot of $\delta_{corr,\,Na^+}$ *vs.* temperature describe a line of negative slope (no figure). In Figure 6 (solid lines) we collect $\delta_{corr,\,Na^+}$ *vs.* temperature data for a number of 3 M salt solutions of varying potency as macromolecular destabilizers, and superimpose ribonuclease T_m values for each as above. A scattering of points (filled symbols) rather than a horizontal line is observed. The difficulty can be particularly clearly illustrated by comparing 3 M NaSCN with 3 M NaBr. The lines of $\delta_{corr,\,Na^+}$ *vs.* temperature for the solutions are superimposed (Figure 6), yet ribonuclease melts 34° lower in 3 M NaSCN than in 3 M NaBr. These discrepancies are little improved (Figure 6, dashed lines, open symbols) if we subtract the *total* chemical shift (relative to water) for 3 M NaCl from the chemical shift for the other 3 M salt solutions (see Discussion).

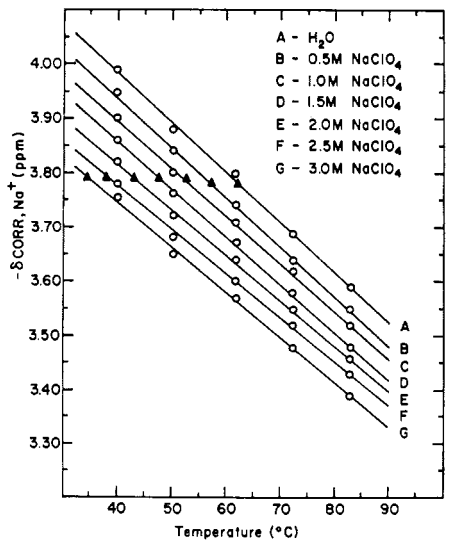

FIGURE 4: The variation of the sodium ion corrected sodium perchlorate induced chemical shift of water protons with temperature in variously concentrated sodium perchlorate solutions. Superimposed on the plot are the transition temperatures of ribonuclease in these same solutions (▲).

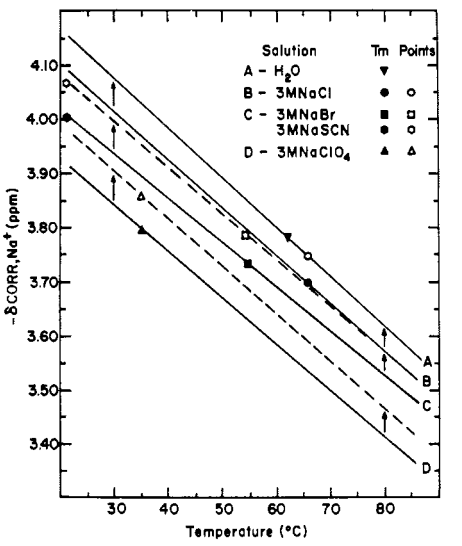

FIGURE 6: The variation of the sodium ion corrected solute induced chemical shift of water protons with temperature in various 3 M sodium salt solutions (—); (---) the same data after adjustment for the shift induced by an equivalent concentration of chloride ion. Superimposed on the plot are the transition temperatures of ribonuclease in these same solutions.

Discussion

The magnitude of the chemical shift of the water protons in aqueous solutions is a sensitive function of environmental conditions. In pure water ("0" M line in Figures 1–6) a progressive upfield trend (toward less negative chemical shift values) is observed with increasing temperature. This trend indicates that the magnitude of the magnetic field required to induce resonance of the average water proton increases with increasing temperature, due to a progressive increase in the diamagnetic shielding of the average water proton. Such increases in shielding are generally attributed to hydrogen bond breakage, with increased electron density around the proton resulting from the removal of the electronegative oxygen which had previously served as the hydrogen bond acceptor; or to hydrogen bond distortion (for details and references, see Hindman, 1966, von Hippel and Schleich, 1969a, and Eisenberg and Kauzmann, 1969). In either case, this temperature-sensitive upfield movement of the proton resonance for liquid water reflects the disruption or "loosening" of water structure with increasing temperature.

The addition of ions to the water further complicates the situation by providing a number of additional magnetic environments for the water proton. Thus some water protons will find themselves immediately adjacent to an ion, and thus relatively immobilized and polarized in the ionic electrostatic field. This electrostatic polarization leads to a downfield shift for protons in the vicinity of both cations and anions, though the magnitude of the shift is not the same for both because of differences in water–ion geometry. (In cation–water complexes the protons are directed away from the surface of the ion, while in anion–water complexes the opposite is true.) Charge redistribution effects induced by highly dipolar ions (such as SCN^-) should induce an additional downfield component (*e.g.*, see Hindman, 1962). Electrostatic polarization effects should be most marked for small ions (*e.g.*, Li^+, Ca^{2+}) with intense electrostatic fields at the ionic surface. Large ions have lower surface charge densities, and thus disrupt the water lattice in their vicinity without orienting and immobilizing the surface layer of water molecules to the same extent.

We will not discuss in further detail the various contributions to the water proton chemical shift which must be considered in aqueous electrolyte solutions (for further discussion and references see, for example, Hindman, 1962, 1966, and von Hippel and Schleich, 1969a). Suffice it to say that some of these contributions (such as the electrostatic polarization components) might be quite temperature insensitive, while others will be strongly temperature dependent. In general, the temperature dependence of the water-proton chemical shift is decreased by the addition of electrolytes (see Table I), though the magnitude and concentration dependence of this decrease depends on the specific ions present.

As a simple first approximation model, let us suggest that ions of the type which disrupt water structure without appreciable dipolar water-binding will show a temperature dependence of the water proton chemical shift which is identical with that of water, but moved progressively upfield from the water line with increasing salt concentrations (*e.g.*, Figure 3, for increasing concentrations of ClO_4^-). This is the sort of situation in which the chemical shift can be used as a measure of water structure temperature in the Bernal–Fowler sense. Appreciable dipolar water binding should decrease $d\delta_{corr}/dT$ as the salt concentration increases (*e.g.*, see Figures 1 and 2, for NaCl and LiCl), and this progressive increase in the magnitude of the temperature-independent component of the proton chemical shift makes a pure "structure temperature" interpretation less suitable.[3]

Much has been written about the role of water structure in controlling the stability of macromolecular conformations, and how changes in water structure induced by various destabilizing electrolytes might therefore be responsible for their destabilizing effects (for recent reviews and references, see von Hippel and Schleich, 1969a,b). However, briefly stated, it seems clear that most of the stability of the folded (native) structures of proteins (and probably nucleic acids as well) depends on the unfavorable thermodynamic consequences of transferring nonpolar groups from the interior of the macromolecule into contact with the solvent. And at least a part of this positive free energy of transfer is due to an increase in water structural organization around the newly exposed groups. Compared with non-water-structure-perturbing ions, ions which do disrupt water structure would tend (like increased temperature) to disorganize the structures induced about the exposed groups, and thus destabilize the "native" macromolecular conformation relative to the melted form by reducing the magnitude of the positive free energy of transfer of these groups into the solvent. This is consistent with the observation that water structure perturbing ions are less effective than nonperturbing ions in salting-out nonpolar groups from aqueous solution (for further discussion and references, see von Hippel and Schleich, 1969a,b).

If a particular ion destabilizes macromolecular conformations primarily through effects on solvent structure as indicated above, and *if* its effects on water are simply to isothermally increase the disruption of the water structure without introducing new forms of water organization, then we may expect to see correlations between ion-induced water-proton chemical shifts and T_m effects such as those of Figure 4 for ClO_4^-. Furthermore it is not surprising, as shown here, that if the correlation works for one macromolecule it will work for others, since (as stated above) the molar effectiveness of a given ionic destabilizer is very similar for the various macromolecules which have been examined. That is, T_m points for DNA could be put onto Figure 4, and would form a horizontal line starting at about 90° on the "0" M line, while collagen T_m data would form a horizontal line starting at about 30° on the "0" M line. On the other hand, *if* the ion has additional effects on water structure, or *if* the ion affects macromolecular stability by some other mechanism (*e.g.*, direct binding) in addition to water perturbation, the correlation breaks down.

These difficulties can be seen qualitatively by inspecting Figures 1 and 2. NaCl (Figure 1) has essentially no effect on macromolecular stability. Thus in terms of the simple water structure correlation all the lines of δ_{corr} *vs.* temperature for different NaCl concentrations should be superimposed on the "0" M line. This is clearly not the case, and indicates that

[3] Malinowski *et al.* (1966) and Creekmore and Reilley (1969) have made use of measurements of this temperature-independent component of the chemical shift, plus certain simplifying assumptions, to measure the hydration number of various ions.

other effects are operative on the chemical shift. LiCl (Figure 2) is a moderately effective T_m destabilizer for all macromolecules examined, yet a straightforward water structure interpretation of Figure 2 would suggest (contrary to known fact) that this salt is an effective destabilizer at lower temperatures, essentially ineffective around 45°, and an effective *stabilizer* of macromolecular conformation at high temperatures.

Water-proton chemical shift effects, like T_m effects, can be dissected into ionic components. This is done by choosing an ion which a number of independent criteria suggest has little or no effect on water structure, and assigning it a chemical shift value of zero. Hindman (1962) chose NH_4^+ for this purpose, and assigned ionic water-proton chemical shift values on this basis. He clearly demonstrated (see Table II in Hindman, 1962) the additivity of these ionic shift values by showing that the same values were obtained for each common ion in a number of sets of different uniunivalent salts. Furthermore, his results showed that these ionic water-proton chemical shift values are essentially independent (per mole) of salt concentration, since the chemical shift for most salts tested was found to be quite linear with salt concentration up to concentrations of 5 *m* and above. We have used Hindman's value of the Na^+ chemical shift (assumed temperature independent) to make the sodium ion corrections to the measured chemical shifts in Figures 3–6 (for details of the calculations, see Gordon *et al.*, 1965).

On this basis the effects of Cl^- on the water-proton chemical shift turn out to be quite small, and most of the displacements from the "0" M line in Figures 1 and 2 are due to Na^+ and Li^+, respectively. This shows that a T_m correlation of the ClO_4^- type (Figure 4) will not work for destabilizing cations such as Li^+, and further suggests that the correlation should not work for destabilizing anions until the chemical shift had been corrected for the Na^+ contribution. Figures 3 and 4 bear out this expectation. Figures 5 and 6 show, furthermore, that a good correlation is not obtained for other anions even after the Na^+ correction has been made, or (Figure 6) after the entire chemical shift from the "0" M line due to nonstructure perturbing NaCl has been subtracted.

We may conclude then, in keeping with other types of evidence (see von Hippel and Schleich, 1969a), that the perchlorate anion does indeed effect macromolecular stability through water structure perturbation, and that the extent of this perturbation is measurable through determination of the ClO_4^--induced water-proton chemical shift. (Of course we could also attempt to attribute the perchlorate result to a number of fortuitously cancelling effects.) Other anions have additional effects on the chemical shift, or macromolecular stability, or both, though the observed trends suggest a perchlorate-like component of the effects of the other anions tested. In fact, we could rationalize corrections to the anion data which would make the correlation considerably better, *e.g.*, in Figure 6 we could easily justify the lowering of the $\delta_{corr,\,Na^+}$ *vs.* temperature line for SCN^-, due to the highly dipolar character of this ion (see above) and thus bring the T_m points for SCN^- more into line with the others. However, such manipulation of the chemical shift data becomes quantitatively rather uncertain because of our lack of detailed knowledge of the properties of electrolyte solutions and of how these properties might be reflected in the overall chemical shift. And as long as such knowledge is lacking it appears that a satisfactory *general* correlation between a measured physical parameter of a number of electrolyte solutions, and the effectiveness of these electrolytes in altering macromolecular stability, will continue to elude us.

Acknowledgments

Most of the experimental work reported in this paper was performed while the authors were members of the Biochemistry Department, Dartmouth Medical School, Hanover, N. H. We are very grateful to Professors Paul R. Shafer and Karl Kuhlmann of the Dartmouth Chemistry Department for making their nuclear magnetic resonance equipment (supported by a grant from the National Science Foundation) available to us.

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Circular Dichroism Studies on the Acid Denaturation of γ-Immunoglobulin G and Its Fragments*

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ABSTRACT: Circular dichroism of human immunoglobulin G and its tryptic digestion fragments Fab(t) and Fc(t) was studied in neutral and acid solutions. In neutral solutions, all the proteins examined showed at least two circular dichroism bands in the far ultraviolet zone: a negative band centered at 217 mμ and a positive band at approximately 202 mμ. The former indicates the presence of the β structure. In hydrochloric acid (pH 2.2), the positive band at 202 mμ was shifted to a negative band at 200 mμ, whereas the negative band at 217 mμ was affected very little. At low pH, all the other bands (at 230 to 300 mμ) were strongly reduced. The negative ellipticity values at 200 mμ in acid (pH 2.2) were different for each protein but the general features of the circular dichroism curves, including those of more or less completely renatured proteins, were similar. Lower ionic strength favored disorganization and separation of the polypeptide chains in acid solution, as confirmed by sedimentation and viscosity data.

The results led us to conclude that disorganization by acid and separation of the chains were caused by electrostatic repulsive forces which overcame other interactions, such as hydrophobic and hydrogen bonds. Also, it was concluded that the disorganization in acid was incomplete and that the circular dichroism spectra were determined to a large extent by unspecified constrained polypeptide backbone conformations and amino acid side chains.

The optical rotatory dispersion of immunoglobulin G has been studied in several laboratories. Analyses by Drude and Moffitt methods have been interpreted to indicate little or no α helix in IgG[1] (Jirgensons, 1958, 1961; Winkler and Doty, 1961; Imahori and Momoi, 1962; Hamaguchi and Migita, 1964; Gould *et al.*, 1964). Several investigators have suggested that some β structure may be present in native γ-globulin (Callaghan and Martin, 1963; Imahori, 1963). Cotton effects of normal human IgG in the far ultraviolet led to the conclusion that some β structure was present (Jirgensons, 1965, 1966a,b, 1969).

Analysis of the circular dichroism of rabbit IgG by Sarkar and Doty (1966) showed a negative band near 217 mμ. The position of this CD band corresponds to that found for poly-L-lysine, poly-L-serine, and silk fibroin in the β conformation (Sarkar and Doty, 1966; Townend *et al.*, 1966; Iizuka and Yang, 1966; Quadrifoglio and Urry, 1968; Li and Spector, 1969). The same observations have been reported recently for human IgG and Bence-Jones proteins (Ross and Jirgensons, 1968; Ikeda *et al.*, 1968) and for rabbit IgG and its papain fragments (Cathou *et al.*, 1968). The positive band in the deep ultraviolet at 200–202 mμ was first reported by Ross and Jirgensons (1968). The gross conformation of papain fragments Fab and Fc has been described by Noelken *et al.* (1965). They concluded that the fragments existed in the parent molecule in the same conformation as when isolated from it.

The present work offers data on the circular dichroism spectra of human IgG and its tryptic digestion fragments, Fab(t)[1] and Fc(t), in the native state and acid-denatured state. The changes in the circular dichroism spectra of the Fc(t) fragment were studied in detail and the results were compared with those of the Fab(t) fragment, parent IgG, and myeloma IgG.

Materials and Methods

Ultra Pure guanidine hydrochloride was obtained from Mann Research Laboratories (New York, N. Y.) and was used without further purification. Iodoacetamide (Mann Re-

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[1] The abbreviations used are: IgG, Fab, and Fc, as recommended by the World Health Organization (1964); Fab(t) and Fc(t) are used for the tryptic fragments similar to the papain digestion fragments Fab and Fc; Gu·HCl, guanidine hydrochloride.