acetal hydroxyl of the reducing end to the core phosphate to form a phosphodiester linkage branch point. The nonreducing termini of this proposed polymer structure would then be nonphosphorylated pentasaccharide units, and depending on the degree of branching in the polymer, may be present in quite significant amounts. Fragment 4 of Figure 1 may represent or contain such terminal oligosaccharide units as very little phosphate is present in this fraction. A more detailed structural analysis of this fragment is currently being carried out.

Our preliminary observation of 1→6′ linkages in the core fragment is of interest because of the common occurrence of a 1→6′-linked backbone in yeast cell wall mannans (Stewart and Ballou, 1968; Kocourek and Ballou, 1969). Whether any biosynthetic similarities or precursor–product relationship exists between the exocellular phosphomannans and cell wall mannans remains to be determined.

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Model Studies on the Effects of Neutral Salts on the Conformational Stability of Biological Macromolecules. I. Ion Binding to Polyacrylamide and Polystyrene Columns†

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ABSTRACT: Measurements are reported of the binding constants to polyacrylamide gels of various neutral salts that can serve as perturbants of the stability of macromolecular conformations. Recycling chromatographic methods are used to obtain accurate values of these small numbers. It is shown by comparative measurements on polystyrene columns that binding occurs only to the amide moieties, and that ions are neither selectively attracted into, nor excluded from, the hydration shell surrounding nonpolar groups. Binding constants ($K_{a,rel}$) are defined *relative* to the binding of a tritiated water tracer, those measured for macromolecular conformation destabilizing ions showing positive values of $K_{a,rel}$, and those measured for conformation-stabilizing ions yielding negative $K_{a,rel}$ values. Single-ion binding constants, and values of ΔH° and ΔS° for the binding process, are also defined and determined. It is shown that the acrylamide moiety has a polar/nonpolar ratio comparable to the average group exposed in a protein as a consequence of a thermally induced unfolding process, and values are calculated for the free energy of transfer (per acrylamide unit) from water to various stabilizing or denaturing salts. These values can then be used to calculate the change in free energy stabilizing the native conformation of a protein which accompanies the transfer of the macromolecule into a conformation-perturbing solvent of given composition, when the change in peptide group exposure on unfolding is known. Alternatively, using melting temperature depression data, these values can be used to calculate the net number of amide groups exposed in transitions for which the molecular details are not known.

A considerable body of research over the past 10 or more years has established beyond doubt that concentrated solutions of various neutral salts have profound effects on the conformational stability of a variety of biological macromolecules and macromolecular assemblies. In essence, the effect may be viewed as a shifting, by the added salt, of the transition boundary between an ordered macromolecular structure (in which residue–residue contacts are thermodynamically favored for at least the groups comprising the "interior" of

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the macromolecule) and a disordered or "random" coil state (in which residue–solvent contacts are favored for essentially all of the constituent groups). The phase transition boundary may be shifted toward either higher or lower temperatures relative to the transition temperature (T_m) of the macromolecule in a dilute aqueous buffer system, depending on whether the added ions tend to stabilize (thermodynamically favor) the ordered or the disordered forms of the macromolecule. The effects of the individual ions on macromolecular stability are independent and additive, and generally follow the classical Hofmeister series. The present status of the field has recently been reviewed (von Hippel and Schleich, 1969a,b).

Having shown *what* effects a particular neutral salt solution can be expected to have on the stability of a folded macromolecule or the residue–residue interactions involved in holding together a macromolecular assembly, various workers have turned to the question of *how* these effects are brought about. Studies on macromolecules have made it clear that the effects do not depend strongly on details of macromolecular conformation or chemistry. Therefore the major research emphasis has now turned to an examination of the properties of small molecule models of macromolecular functional groups in aqueous solutions of concentrated neutral salts. These studies may be divided into a "thermodynamic" group, in which the effects of neutral salts on the solubility (*i.e.*, activity coefficient) of model compounds have been measured (*e.g.*, Robinson and Jencks, 1965; Robinson and Grant, 1966; Schrier and Schrier, 1967; Nandi and Robinson, 1972a,b) and "mapping" studies in which the molecular sites and mechanisms of binding of the various ions have been examined (*e.g.*, Bello and Bello, 1961a,b; Bello *et al.*, 1966; Schleich *et al.*, 1968, 1971).

The thermodynamic studies have focused mostly on model compounds containing one or more peptide groups surrounded by various protein side-chain analogs. These represent typical groups which would be expected to be found on the "inside" of folded proteins. Neutral salts which increase the solubility of such species in the aqueous environment would be expected to decrease the free energy of stabilization of the ordered (folded) form of biological macromolecules, while neutral salts which decrease the solubility of the model species would be expected to act as macromolecular conformation stabilizing agents. Studies on the solubility of mononucleosides and nucleoside bases in aqueous solution (Robinson and Grant, 1966; von Hippel and Chang, manuscript in preparation) show that qualitatively similar effects may be observed with these moieties, suggesting that the "stacking" interactions which help to stabilize the ordered form of the various nucleic acid conformations are similarly affected by neutral salts.

Schrier and Schrier (1967) analyzed their solubility data and those of Robinson and Jencks to suggest that the concept of additivity could be applied to the molecule being subjected to "salting-in" or "salting-out" in the model system, as well as to the ions responsible for the effect. Thus, they proposed that the salting-out coefficient for a particular system could be analyzed into contributions from the functional groups comprising the molecule whose solubility was under test. Their results suggested that mono-monovalent ions have a relatively constant salting-in effect on the solubility of the amide group in these systems, while the specificity of the interaction of these salts with the varying model systems could be attributed to an ion-specific salting-out effect on the nonpolar constituent groups of the test molecule. One of the major aims of this and the accompanying articles is to report experiments in which this concept is tested and extended to the molecular level by measuring directly the binding of various neutral salts to models of functional groups typical of the protein interior.

The various salting-out studies cited above had clearly demonstrated that the equilibrium constant for the binding of neutral salts to amides in aqueous solution must be very small ($K_a \ll 1\ M^{-1}$). Therefore, conventional methods of measuring binding constants, such as equilibrium dialysis, could not easily be applied. We thus turned to the amplification inherent in the methods of column chromatography, and have measured the binding of ions to amide and nonpolar groups using the technique of recycling chromatography on polyacrylamide and polystyrene columns. The binding constant of interest is the *relative* affinity of these groups for neutral salts, as compared to their affinity for water. For this reason we have measured binding relative to that of a tritiated water marker. It is shown that conformation-stabilizing ions bind to polyacrylamide with a *negative* relative binding constant, while the destabilizing ions show *positive* relative binding. Thus in their relative affinity for polyacrylamide gels the neutral salts again appear to follow the Hofmeister series.

A preliminary report of this rank ordering of the affinity of the neutral salts for polyacrylamide had been presented by us previously (von Hippel and Schleich, 1969b). Similar observations, also in the context of effects on protein denaturation, have been made independently by St. Pierre and Jencks (1969). Such measurements have also been carried out by Egan (1968) and Saunders and Pecsok (1968), though these workers interpreted the results differently (see Discussion).

In this paper measurements of relative ion-binding constants to polyacrylamide columns are extended to several temperatures, permitting the determination of thermodynamic parameters and thus providing additional insight into mechanism. It is also shown that only ions with appreciable nonpolar character will bind (relative to the water marker) to the totally nonpolar polystyrene resin, demonstrating directly that the binding of most neutral salts is to the amide dipole itself, though the nature and magnitude of the binding are presumably "modulated" by the vicinal nonpolar groups. In the following (accompanying) article (Hamabata and von Hippel, 1973), we demonstrate directly how the binding constants are affected by the number and distribution of methyl and methylene groups around the amide dipole.

Materials and Methods

Column Materials. The polyacrylamide gels used in these studies were obtained from Bio-Rad Laboratories (Richmond, Calif.) in dry form as P-100 (50–100 and 100–200 mesh) particles. A sample of P-30 (100–200 mesh) was also used. These materials contain no bound charges. The gels were hydrated by swelling overnight prior to use, and put through six decantation cycles to eliminate "fines." Packed hydrated bed volumes of ~17 ml/dry g were measured for the P-100 gels at room temperature. The bed volumes decreased to ~14 ml/dry g at 4°. The aqueous gel slurries were degassed under vacuum prior to column packing to prevent air-bubble formation in the columns and to remove air from within the beads.

Polystyrene columns were made using either SM-1 or SM-2 porous polystyrene Bio-Beads (cross-linked with divinylbenzene), 20–50 mesh, also obtained from Bio-Rad. These gels were prepared for use by swelling in methanol, followed by thorough water-washing, degassing, and six

"fining" cycles. Packed (hydrated) volumes of ~2 ml/dry g were obtained with these materials at room temperature.

Some runs were also made with dextran beads (Sephadex G-75) obtained from Pharmacia (Uppsala, Sweden). These beads were hydrated, washed, and "fined" as described for polyacrylamide (above) and gave packed hydrated column bed volumes of ~15 ml/dry g at room temperature.

Preliminary experiments were conducted with several nylon samples obtained from various industrial sources. The most successful involved Nylon-4, obtained as a fine powder from General Aniline and Film Corporation, and packed into columns without further processing.

Chromatographic Procedures. The columns were set up as closed systems suitable for recycling chromatography (upward flow); 0.9 × 166 cm columns were used, and siliconized (Siliclad) prior to packing. The columns were loaded under flow, and both ends sealed with porous polyethylene disks. The systems were pumped with LKB variable-speed peristaltic (Recychrom) pumps, usually at flow rates of ~0.5 ml/min. Various types of LKB and other sample injection valves were used, and after the predetermined number of cycles the effluent was collected (usually as 2-ml fractions) in a Gilson fraction collector used in the drop-counting mode. The columns were thermostated (to ±1°) by circulating ethylene glycol through temperature-control jackets, and were packed and continuously maintained at the temperature at which they were to be run. In most runs we used 1-ml samples, usually 0.1 M in the salt being tested, and also containing 0.25 μCi of tritiated water (New England Nuclear) as an internal reference marker. Sometimes two salts (with a common cation or anion), for which different assays were available and which were expected to undergo good separation, were injected as a single sample, or two samples were injected into a single column, approximately one-half cycle apart. Occasionally a dilute solution of $Co(NH_3)_6Cl_3$ was also added to the sample, to serve as a visible (blue) sample position marker. All the salts migrated independently, without intersample interference, in the mixed samples. Elution was generally with distilled water, though occasionally a dilute phosphate buffer was used when the state of ionization of the salt (*e.g.*, sodium benzoate or phenol) might be affected by pH changes. In most experiments 1 ml of 3% sucrose was injected directly behind the sample to provide a positive density gradient during upward flow in the column. The sucrose had no effect on the peak position of the salts. Control runs were also made at varying pumping speeds, to assure that equilibrium was being attained in the columns. Peak positions were found to be independent of pumping speed over the entire range tested (~0.1 to ~1 ml/min).

Assays. Tubes were assayed for salt concentration using a Radiometer CDM2 conductivity meter equipped with a type CDC-114 cell. Assays were also performed with the appropriate Orion specific ion electrodes connected to an Orion Model 404 specific ion meter when two salts were run together. Phenol and $Co(NH_3)_6Cl_3$ were also measured spectrophotometrically, and tritiated water (THO) was determined in a Packard liquid scintillation counter using a dioxane-based counting fluid formulation (Bray, 1960).

Bromination of Polystyrene Beads. In order to determine the styrene groups accessible to the aqueous solvent in the Bio-Beads, the fraction of these moieties subject to bromination of the aromatic ring was measured at room temperature, following a procedure modified from Brown and Stock (1957). Approximately 2-g aliquots of polystyrene (SM-1 or SM-2) beads were weighed into tubes containing ~16 ml of 85% (v/v) acetic acid. The beads were degassed, and then ~2 ml of Br_2 liquid was added to each tube at zero time. (Br_2 was omitted from the control.) The final solutions contained ~0.05 M Br_2 and ~0.015 M styrene units. The tubes were sealed, swirled occasionally to ensure mixing, and at predetermined times the bromination reaction was stopped by removing the unreacted Br_2 (and the Br^- generated in the reaction) by washing the beads with water until the wash liquid was colorless, and then adding $Na_2S_2O_3$ to reduce any Br_2 remaining bound to, or trapped within, the beads. The latter reaction was conducted at alkaline pH to prevent the precipitation of elemental sulfur, and was carried out, with stirring, overnight. Any Br^- which might have remained in the beads was purged by washing with dilute solutions of sodium acetate, and then with water until the conductivity of the eluate had decreased to distilled water values. The aliquots of beads were dried overnight in an Abderhalden pistol at 100° over P_2O_5, weighed, and analyzed for Br by the method of Dixon (1969), involving a final determination by potentiometric titration.

Results

Polyacrylamide. Figure 1 shows the elution profile of a typical five-cycle run made on P-100 polyacrylamide at 25.2°, containing two salts (NaF and NaI) and a tritiated water (THO) marker. As Figure 1 suggests, peak position can be measured with an accuracy of ~±1 ml. We will generally report salt peak positions (in milliliters/cycle) relative to the THO (water) position: a minus volume corresponds to elution *ahead* of THO and a plus volume to elution *behind* the water marker.[1] Thus, in Figure 1 NaF leads the reference by 19 ml, corresponding to a corrected volume/cycle (ΔV_c) of −2.3 ml/cycle. NaI lags the THO peak by 18 ml, corresponding to a ΔV_c of +5.1 ml/cycle. Experiments were generally repeated two–four times, using the number of cycles needed to reduce the standard error in the final value of ΔV_c for a particular salt to <±10%. The results of a large number of experiments like those shown in Figure 1, reported as average values of ΔV_c relative to an internal THO reference, are summarized in Table I.

Approximate relative equilibrium constants for the binding reaction may also be calculated from these data, it having been demonstrated that local diffusional equilibrium is attained in the column by showing that ΔV_c is independent of flow rate. Bio-Gel P-100, with an exclusion limit of ~100,000 daltons, was used in these studies in order to avoid possible gel filtration effects (*i.e.*, exclusion of the ions from any part of the gel). Thus the chromatography being conducted here should be viewed as pure adsorption (see also the Discussion for additional confirmation of this assumption).

To calculate equilibrium constants we assume that the tritiated water tracer exchanges with, and has access to, all

[1] Some retardation of the THO peak on the polyacrylamide gel results from the exchange of the tritium tracer with the nitrogen-bound amide protons of the gel matrix. Exchange of these protons with solvent hydrogens should be "instantaneous" at neutral pH and on the time scale of these experiments, and so (assuming no exchange isotope effect) the retardation of the THO marker due to the presence of these amide hydrogens should be equal to the ratio of the molarity of the amide hydrogens to the water hydrogens in the column (~1.7/111 ≃ 0.015; see text). This corresponds to a ΔV_c of ~+1.5 ml/cycle for the THO marker itself relative to an "idealized" THO marker in the absence of exchangeable hydrogens (for a total column volume of ~100 ml), and thus all the ΔV_c data obtained on polyacrylamide P-100 have been corrected for this effect by adding +1.5 ml/cycle to the measured values prior to calculating relative equilibrium constants, etc.

TABLE I: Relative Binding of Neutral Salts to Polyacrylamide Gels at 25°.[a]

Salt	ΔV_e (ml/cycle)[b]	$K_{a,rel}$ (M^{-1})[c]
NaF	−2.3	-2.8×10^{-2}
NaCl	+0.5	$+0.6 \times 10^{-2}$
NaBr	+2.7	$+3.2 \times 10^{-2}$
NaI	+4.7	$+5.6 \times 10^{-2}$
$NaNO_3$	+3.3	$+4.0 \times 10^{-2}$
$NaClO_4$	+5.9	$+7.1 \times 10^{-2}$
NaSCN	+4.7	$+5.6 \times 10^{-2}$
Na_2SO_4	−0.7	-0.8×10^{-2}
LiCl	+1.1	$+1.3 \times 10^{-2}$
KCl	+0.1	$+0.1 \times 10^{-2}$
RbCl	+0.7	$+0.8 \times 10^{-2}$
CsCl	+1.1	$+1.3 \times 10^{-2}$
$MgCl_2$	+4.1	$+4.9 \times 10^{-2}$
$CaCl_2$	+4.5	$+5.4 \times 10^{-2}$
$BaCl_2$	+4.7	$+5.6 \times 10^{-2}$

[a] For experimental details see text. [b] Displacement (per cycle) of elution peaks relative to the position of the corrected tritiated water marker. Calculated from two–five repeats of (mostly) five-cycle runs. Average standard error in ΔV_e is $\sim \pm 10\%$. See text for further experimental details. [c] *Relative* equilibrium (association) constant for binding ions to amide dipoles of polyacrylamide matrix. See text for assumptions involved in the calculation.

the hydrated loci in the column. To the extent that the column contains bound (transiently immobilized) water molecules, these will exchange with the THO, and so the (corrected for amide group exchange) THO peak is retarded in proportion to the fraction of the total column water which exists in such a "bound" state. Then solutes which bind more tightly than water to the gel matrix show a positive ΔV_e, while ions which compete less effectively than water for binding sites show negative values of ΔV_e.

The bed volume of the P-100 gel at 25° is ~17 ml/g, and therefore a 0.9 × 166 cm column contains ~6 g of polyacrylamide or ~0.09 mol of acrylamide units (–$CH_2CHC(=O)NH_2$–).[2] This corresponds to a concentration of ~0.85 M amide groups. The sample is typically injected at a salt concentration of 0.1 M, and is diluted at least tenfold at the peak position during the run. Thus the amide concentration is always in local excess during a run, and (assuming ion binding is to the amide groups, see below) ΔV_e should be independent of salt concentration. Control experiments demonstrating this directly have been carried out. For example, runs with NaI on polyacrylamide at 25° at initial sample concentrations of 0.01, 0.03, and 0.1 M showed values of ΔV_e of +7.5, +5.5, and +7.5 ml/cycle, respectively.

Based on the above considerations, and assuming that each amide moiety comprises a binding site (A) for an ion pair

[2] Actually, Bio-Gel is made by the copolymerization of acrylamide ($H_2C{=}CHCONH_2$) with the cross-linking agent *N,N'*-methylenebisacrylamide ($H_2C{=}CHCONHCH_2NHCOCH{=}CH_2$), in an input molar ratio (for P-100) of ~40:1. Thus, ~5% of the amide groups in these gels occur in the cross-linked rather than in the free acrylamide form.

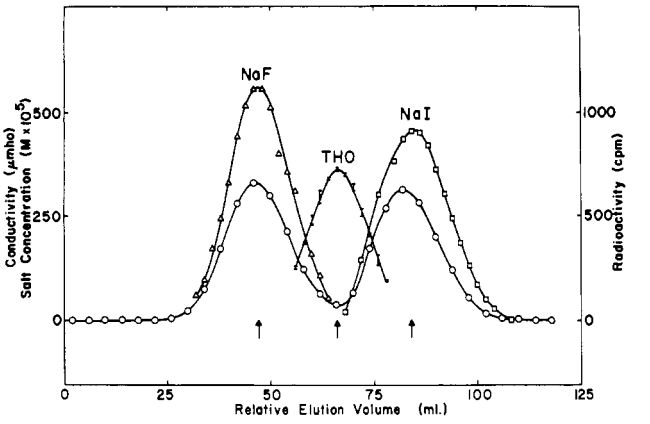


FIGURE 1: Elution profile of 0.1 M NaI, 0.1 M NaF, and 0.25 μCi of tritiated water, injected as a 1-ml sample onto a 0.9 × 166 cm P-100 polyacrylamide gel column: five cycles; 25.2 ± 0.5°; flow rate = 0.4 ml/min; (○) conductivity; (□) iodide electrode; (△) fluoride electrode; (I) tritiated water counts (error bars).

(M^+X^-, or MX), we define a salt–amide association constant (setting all activity coefficients to unity) as

$$K_a = \frac{[A\text{–}MX]}{[A][MX]} \quad (1)$$

In addition, $[A] \simeq [A]_0$ (the total amide concentration) since for this system $[A] \gg [MX\text{–}A]$. Therefore [A–MX]/[MX] is constant and independent of both initial salt concentration and changes in salt concentration during chromatography. We also define a *relative* binding constant ($K_{a,rel}$) based on the internal THO marker as a reference (*i.e.*, $K_{a,rel} \equiv 0$ for salts characterized by $\Delta V_e = 0$), as follows

$$K_{a,rel} = \frac{\Delta V_e}{[A]_0 V_T} \quad (2)$$

where V_T is the total volume of the recycling system available to water or added solutes. For the column configurations used in this study we estimate $V_T \simeq 100$ ml, and, for Bio-Gel P-100 at room temperature, $[A]_0 \simeq 0.85$ M. Equation 2 only applies under conditions where $[A]_0 \gg [MX]_0$, and may be rationalized by considering $K_{a,rel}$ as a (relative) distribution ratio (normalized to the actual amide concentration present) between salt bound to the fixed amide sites and salt free in solution at any particular level in the column. (This ratio, of course, will be independent of the actual local salt concentration as long as amide sites are everywhere in local excess.) Thus, for a hypothetical salt which is half-bound and half-free under these conditions, it will emerge one column volume behind the THO peak (*i.e.*, $\Delta V_e = V_T$), and so $K_{a,rel}$ would be equal to $1/[A]_0$. Values of $K_{a,rel}$ calculated on this basis are included in Table I.

To determine standard enthalpies and entropies of binding, we have measured ΔV_e for six salts (NaF, NaI, $NaClO_4$, NaSCN, Na_2SO_4, and $CaCl_2$) at 10 and 40°, as well as at 25°. These data are summarized in Table II. In order to calculate standard thermodynamic parameters, the values of $K_{a,rel}$ must be converted to absolute equilibrium constants, and this is accomplished by using NaF as a marker of the "unbound" or "front" position (see Discussion). It can be shown that the absolute association constant for each salt

TABLE II: Standard Enthalpy and Entropy Change for the Binding of Neutral Salts to Polyacrylamide.[a]

Salt	T (°C)	ΔV_e (ml/cycle)	$K_{a,rel}$ (M^{-1})	$\Delta H°$ (kcal/mol)[b]	$\Delta S°$ (eu)[b]
NaF	10	−2.5	-3.2×10^{-2}		
	25	−2.3	-2.8×10^{-2}	~0	
	40	−2.7	-3.0×10^{-2}		
NaI	10	+6.9	$+9.0 \times 10^{-2}$		−15.4
	25	+4.7	$+5.6 \times 10^{-2}$	−3.3	−16.0
	40	+3.5	$+3.8 \times 10^{-2}$		−15.9
$NaClO_4$	10	+8.9	$+11.5 \times 10^{-2}$		−13.4
	25	+5.9	$+7.1 \times 10^{-2}$	−2.7	−13.6
	40	+4.7	$+5.2 \times 10^{-2}$		−13.5
NaSCN	10	+7.3	$+9.5 \times 10^{-2}$		−15.8
	25	+4.7	$+5.6 \times 10^{-2}$	−3.3	−16.0
	40	+3.7	$+4.1 \times 10^{-2}$		−15.8
Na_2SO_4	10	+0.7	$+0.9 \times 10^{-2}$		−24.8
	25	−0.7	-0.8×10^{-2}	−5.2	−25.1
	40	−1.3	-1.4×10^{-2}		−24.8
$CaCl_2$	10	+5.5	$+7.1 \times 10^{-2}$		−8.4
	25	+4.7	$+5.6 \times 10^{-2}$	−1.1	−8.6
	40	+4.1	$+4.5 \times 10^{-2}$		−8.6

[a] Run on Bio-Gel P-100. Temperatures controlled to ±1°. Other experimental details as for Table I. Values of $K_{a,rel}$ were calculated using the value of V_T appropriate to the experimental temperature. [b] See text for basis of these calculations. Because of the various corrections involved, the total errors in the $\Delta H°$ data could be as large as ±1.0 kcal/mol, making the error *between* (though not within) sets of $\Delta S°$ data as large as ±2–3 eu.

(K_a) may then be approximated by $K_{a,rel}$ minus the value of $K_{a,rel,NaF}$ under the same conditions. As Table II shows, $K_{a,rel,NaF}$ is essentially independent of temperature, and is taken as -3.0×10^{-2} M^{-1} for all the calculations of K_a. Values of $\Delta H°$ are then taken from the slopes of the best straight lines fitted through plots of ln K_a *vs.* T^{-1} ($°K^{-1}$), and these $\Delta H°$ values (assumed independent of T over this range) are combined with the calculated values of K_a to determine $\Delta S°$ for each salt and temperature (Table II).[3]

Obviously, for reasons of electroneutrality, the conjugate cation and anion of a salt must migrate together. However, if we apply the principle of additivity of ion effects which has been extensively documented for both macromolecules and model compounds (*e.g.*, see von Hippel and Schleich, 1969a, and also Discussion), the ΔV_e value for a given salt can be taken as the algebraic sum of the contributions of the individual ions. Thus we can define a "single-ion" binding constant scale by arbitrarily assigning $K_{a,rel}$ values to a selected cation and anion. For this purpose we attribute the value of $K_{a,rel}$ for NaCl ($+0.6 \times 10^{-2}$ M^{-1}) equally to each ion, to make $K_{a,rel,Na^+} \equiv K_{a,rel,Cl^-} \equiv +0.3 \times 10^{-2}$ M^{-1}. Single ion values of $K_{a,rel}$ at 25°, calculated in this fashion, are summarized in Table III.

Polystyrene. A series of recycling chromatographic experiments was also conducted with porous polystyrene beads (Bio-Beads SM-1, exclusion limit ~200 Å), using the same apparatus and procedures described for the polyacrylamide studies. The binding of a number of salts relative to that of water was measured and the results are compiled as ΔV_e values in Table IV. The essential conclusion from these data is that, with the exception of sodium benzoate (which shows strong binding) and un-ionized phenol (which binds so strongly that it cannot be displaced with water at all), all the other salts tested showed elution volumes very close to that of the tritiated water peak (ΔV_e values ranging from −4 to 0 ml/cycle).

Two important conclusions can be derived from these results. First, only ions with very nonpolar (and here aromatic) character show measurable binding to the nonpolar polystyrene matrix. This confirms the above assumption that the major binding sites on the polyacrylamide gels are the amide dipoles, since both polymers have an identical methylene backbone structure. And second, there is no significant organized water layer or shell around the nonpolar matrix from which ions are preferentially excluded.

As before, the tritiated water tracer should come into equilibrium with all the water (bound and unbound) in the column. The polystyrene spheres do not swell appreciably, and so (compared to the polyacrylamide gels) the concentration of gel material in the column is quite high (~53 g per column volume). This means that the concentration of styrene units ($CH_2C(H)C_6H_5$) in the column is ~5 M. If we (conservatively) estimate that each styrene unit exposed to the solvent has 10–15 nearest-neighbor water molecules, and if these water molecules were organized into a hydration layer *impenetrable to ions*, the neutral salts should emerge from the polystyrene columns at very large negative ΔV_e values. However, in order to estimate the expected magnitudes of these negative values of ΔV_e, it is necessary to determine the number of styrene moieties in these relatively compact beads which actually *are* accessible to the solvent. (For example, if the beads were solid impermeable spheres ~1 mm in diameter, it can be estimated that only one styrene in $\sim 10^4$ would be exposed on the surface of the beads, and as little as 0.1–1% of the column water would be directly adjacent to a nonpolar moiety, thus completely undermining the experimental basis of the second conclusion listed above.)

The experiments with sodium benzoate and phenol (Table IV) show that these polystyrene matrices *can* adsorb and retain nonpolar compounds from aqueous solution.[4] The binding capacity of SM-1 beads for phenol was measured directly by determining how much phenol could be adsorbed by the beads in a column prior to "break-through" of the front. The measured binding capacity was 0.57 ± 0.01 mol of phenol/mol of styrene, suggesting, if each styrene binds one phenol by a "stacking" interaction, that approximately

[3] In actual fact, all the three-point van't Hoff plots used in determining $\Delta H°$ appear to be somewhat convex downward, indicating that $\Delta H°$ may decrease (becoming numerically smaller) with increasing temperature and thus that $\Delta S°$ also decreases (numerically) more rapidly with increasing temperature than shown in Table II. This phenomenon is just marginally outside the limits of error of the measurements, but appears consistently.

[4] The runs with sodium benzoate were carried out in dilute phosphate buffer (pH 7) to assure that the benzoic acid ($pK_a \sim 4$) was totally ionized. Steinberg and Scheraga (1962) have previously shown that partially ionized long-chain fatty acids can be adsorbed onto porous polystyrene particles and differentially displaced with water, with the shorter chain acids coming off first. Since the experiments were conducted in water, the state of ionization of the bound molecules was undefined.

TABLE III: "Single-Ion" Parameters for Binding of Ions to Polyacrylamide at 25°.[a]

Cation	$K_{a,rel}$ (M^{-1})	$\Delta(\Delta G_{tr})_{res}$ (cal/mol)[b]	Anion	$K_{a,rel}$ (M^{-1})	$\Delta(\Delta G_{tr})_{res}$ (cal/mol)[b]
K^+	-0.2×10^{-2}	+1.2	F^-	-3.1×10^{-2}	+18
Na^+	$+0.3 \times 10^{-2}$	−1.8	SO_4^{2-}	-1.4×10^{-2}	+8.3
Rb^+	$+0.5 \times 10^{-2}$	−3.0	Cl^-	$+0.3 \times 10^{-2}$	−1.8
Cs^+	$+1.0 \times 10^{-2}$	−5.9	Br^-	$+2.9 \times 10^{-2}$	−17
Li^+	$+1.0 \times 10^{-2}$	−5.9	NO_3^-	$+3.7 \times 10^{-2}$	−22
Mg^{2+}	$+4.3 \times 10^{-2}$	−25	SCN^-	$+5.3 \times 10^{-2}$	−31
Ca^{2+}	$+4.8 \times 10^{-2}$	−28	ClO_4^-	$+6.8 \times 10^{-2}$	−40
Ba^{2+}	$+5.0 \times 10^{-2}$	−29			

[a] Based on assignment of the NaCl value of $K_{a,rel}$ equally to Na^+ and Cl^- (see text). [b] Calculated for 25° and 1 M ion. See Discussion for the basis of calculation and the significance of this parameter.

one-half of the styrenes are exposed and accessible to the solvent.

Access of the styrenes to the aqueous solvent was also measured directly by a "chemical probe" technique involving the monitoring of the rate and extent of bromination of the aromatic rings by Br_2 in aqueous acetic acid. The results are shown in Table V, and indicate that the reaction proceeds at a rate roughly comparable to that of the bromination of toluene under the same conditions (compare with Brown and Stock, 1957), reaching a plateau of 0.51 ± 0.01 mol of Br/mol of styrene. No apparent changes in bead geometry (swelling, etc.) were induced by this treatment. Based on one Br per aromatic ring (Brown and Stock, 1957), this result also shows that in these porous polystyrene columns approximately 50% of the styrene units are accessible to the solvent, and thus that the concentration of *exposed* styrene units in the Bio-Bead columns is ~2.5 M.

Using these results we can estimate that at least one-half of the water in the column exists as nearest neighbors to nonpolar groups, and since the total column volume available to THO is ~70 ml, ions excluded from this "primary hydration layer" would be expected to emerge with ΔV_e values of −35 to −50 ml/cycle. This is clearly not observed; in fact, the data of Table IV show (on the basis of this measured level of accessibility of the nonpolar matrix to the solvent) that there is essentially *no* water in the column from which ions are excluded.

Other Column Materials. Some preliminary and qualitative experiments were run with other column materials, with results consistent with those cited above.

Runs made on P-30 Bio-Gel (polyacrylamide), which swells less than P-100 and therefore contains about 30% more amide groups per column volume, did indeed show somewhat increased average values of ΔV_e for several salts, relative to those measured under comparable conditions on P-100.

A few experiments were also carried out with Sephadex (cross-linked dextran) G-75 gels. Essentially no binding (relative to the THO markers) was observed to this matrix, ΔV_e values of −2 to −4 ml being obtained for $CaCl_2$ and LiI, both of which showed appreciable positive ΔV_e values on polyacrylamide. (However, see St. Pierre and Jencks, 1969, who did find some specific binding of ions to Sephadex.)

Originally we had intended to conduct these experiments on a series of nylons (monomer structure I) and extrapolate the resulting data to pure "polyformamide" ($n = 0$). We obtained appropriate nylon samples from various industrial sources, but found the problem of converting these materials to forms with the high surface-to-volume ratio needed for effective chromatography to be quite intractable.

TABLE IV: Relative Binding of Neutral Salts to Polystyrene Gels at 25°.[a]

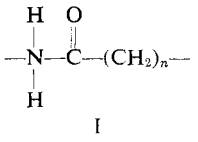
Salt	ΔV_e (ml/cycle)
NaCl	−1
NaF	−2
NaI	−0.5
$(NH_4)_2SO_4$	−1.8
$(CH_3)_4NCl$	0
$(CH_3CH_2)_4NCl$	−3
$(CH_3CH_2CH_2)_4NCl$	−2
$(CH_3CH_2CH_2CH_2)_4NCl$	−4
Sodium formate	−2
Sodium acetate	−3
Sodium propionate	+1
Sodium benzoate[b]	+30
Phenol[b]	Could not elute

[a] Averaged values of one–six experiments each, utilizing both single and five-cycle runs. For further experimental details, see text. [b] Run in, and eluted with, dilute phosphate buffer, pH 7.0.

TABLE V: Bromination of Polystyrene Beads in 85% Acetic Acid.[a]

Reaction Time (hr)	Resin	Mol of Br/Mol of Styrene[b]
45 (min)	SM-1	0.32
24	SM-1	0.43
96	SM-1	0.53
96	SM-2	0.53
168	SM-1	0.49

[a] For reaction conditions, etc., see text. [b] Standard error estimated at less than ±1%.



I

Some moderately successful recycling runs were accomplished with Nylon-4 ($n = 3$ in structure I), with results in general accord with those obtained on polyacrylamide. Thus on Nylon-4 we found LiI to be appreciably retarded relative to the THO marker ($\Delta V_e \simeq +12$ ml), while NaCl emerged prior to the THO peak ($\Delta V_e \simeq -2$ ml). No quantitatively reliable way was found to estimate the accessible binding sites on these samples, and so these studies were not continued.

Discussion

The principal conclusions of this study, and the assumptions involved in reaching them, may be summarized as follows. Neutral salts bind to polyacrylamide with the same rank order as they destabilize (or stabilize) biological macromolecules (compare "single-ion" order indicated in Table III with rankings listed for macromolecular effects, *e.g.*, in von Hippel and Schleich, 1969b). The point in the rankings at which a parameter such as the salting-out coefficient of a model compound switches from negative to positive values (from salting-out to salting-in in an aqueous solution) depends on the ratio of polar to nonpolar groups in the model compound (Schrier and Schrier, 1967). We may note (Table III) that for anions the transition from negative to positive values of $K_{a,rel}$ occurs close to Cl^-, which is exactly the point at which the switch from stabilizing to destabilizing anions comes for proteins (as well as for nucleic acids at high enough salt concentrations to swamp out charge-shielding electrostatic effects on phosphate–phosphate interactions). This suggests that the polar to nonpolar ratio for the acrylamide moiety is very comparable to that of the average protein "interior." The transition from negative to positive values of $K_{a,rel}$ also comes in the appropriate part of the cation rankings, though the small differences in $K_{a,rel}$ for the monovalent cations make the transition point rather dependent on the assignment of K_{a,rel,Na^+} exactly equal to K_{a,rel,Cl^-} used in setting up the single-ion binding constant scale.

As St. Pierre and Jencks (1969) have pointed out, in principle differences in the affinity of a column for ions may be attributed either to actual binding to functional groups on the column, or to differential exclusion from, or attraction to, the neighborhood of the column matrix due to differences in ion activity coefficients in the vicinity of the matrix *vs.* values in the bulk solution. The use of an internal "water marker," plus the experiments with the polystyrene columns, may partially resolve this ambiguity. None of the neutral salts bind to the polystyrene matrix, proving that the binding to the polyacrylamide gels is entirely to the amide moiety. Also, none of the salts were appreciably excluded from the polystyrene beads, *relative to water*, meaning that all the ions tested have essentially the same access as water to the nonpolar column volume. We should specifically note here that this result cannot be inconsistent with the well-known and well-documented fact that different salts vary markedly in their effectiveness in "salting-out" purely nonpolar compounds from aqueous solution. Rather the present finding shows only that the mechanistic basis of these differential salting-out effects is not due to a measurable differential exclusion of the ions from the water immediately surrounding the nonpolar groups.

Since ions showing negative values of $K_{a,rel}$ for polyacrylamide are therefore not excluded from the vicinity of the amide groups by general water structure effects (*i.e.*, "hydrophobic hydration layers") around vicinal nonpolar groups, they must be excluded primarily by their relative inability (at the ion concentrations used) to compete for, and thus displace, the water molecules directly bound to the amide dipole. The results summarized in Table II show that NaF exhibits the largest negative values of ΔV_e of the salts tested. This, coupled with the apparent ΔH°_{rel} value of ~ 0 observed for this salt, suggests that it is almost completely ineffective (at the salt concentrations used) in competing for amide-bound water. Therefore the difference between the column volume available to THO (after correction for amide hydrogen exchange) and that available to NaF can serve as a measure of the "water of hydration" of the amide dipole itself, *defined* as that inaccessible to NaF. In P-100 gels this corresponds to ~ 2.3 ml, or ~ 0.4 g of bound water/g of polyacrylamide (*i.e.*, ~ 2 water molecules/amide group).[5]

We used Bio-Gel P-100 polyacrylamide in these studies to avoid possible molecular sieving in the gel on the basis of ion size. In fact, Saunders and Pecsok (1968) and Egan (1968) have published papers on the use of Bio-Gel for analytical purposes in the chromatography of strong electrolytes, attributing the relative retardation seen largely to molecular sieving effects based on ion size. Using Egan's data obtained with P-2 and P-100 gels, together with the values of hydrated bed volume for the gels (the much more highly cross-linked P-2 gels swell much less), we can calculate directly that the relative retardation of a bound ion (*e.g.*, Ca^{2+}) is directly proportional to the molarity of amide groups in the column, thus supporting our method of calculating $K_{a,rel}$ (eq 2), as well as our assumption of complete availability to solvent of the amide groups in P-100. Egan also demonstrated that the elution volume of a given neutral salt can be resolved into additive cation and anion contributions, providing direct support for the assumptions underlying the presentation of single-ion values of $K_{a,rel}$ in Table III.

In addition to the consequences for the interpretation of the polyacrylamide data, the polystyrene results also have a direct bearing on our views of the nature of hydrophobic bonding and its perturbation by neutral salts. These results demonstrate clearly that nonpolar groups are not surrounded by organized water layers which are impenetrable to ions, and therefore differential exclusion of ions from such hydration layers cannot provide the basis of the differences in salting-out effectiveness (from aqueous solution) of the various neutral salts for nonpolar compounds (*e.g.*, see Long and McDevit, 1952).[6] Rather the explanation must be found in the differences in the structures adopted by the three component

[5] We may note that this value is close to the 0.3–0.5 g/g of protein found for the water of hydration of proteins as defined by the proton nmr freezing technique of Kuntz *et al.* (1969). This technique defines as water of hydration that portion of the water in an aqueous protein solution which does not freeze with the bulk solvent and thus continues to give a relatively narrow water proton nmr signal to $\sim -35°$.

[6] This conclusion is further supported by the finding that the small negative values of ΔV_0 seen for the various salts on polystyrene (Table IV) are approximately the same for salts such as NaF or $(NH_4)_2SO_4$ (which are effective macromolecular stabilizers and nonpolar salting-out agents) and NaI or tetrabutylammonium chloride (which are effective macromolecular destabilizers and much less potent than the above as nonpolar salting-out agents) (see von Hippel and Schleich, 1969b).

water–nonpolar group–salt systems in realizing an overall free-energy minimum configuration in the presence of various types of ions and nonpolar groups. See Hamabata and von Hippel (1973) for further discussion of this point.

The thermodynamic parameters calculated in Table II refer specifically to the process of binding of the ions to the amide moiety in water solution. Thus the negative values of ΔH° obtained for the mono-monovalent salts show that the process of binding ions to the amide dipole is enthalpically favorable relative to leaving both amide and ions in their separate hydrated states. The negative values of ΔS° show that this favorable enthalpy is more than offset by a net ordering of the system (increase in water structure?) as a consequence of ion binding, presumably because the net water structure perturbing effects of the free ions exceeds that of the ions in the bound form. Both ΔH° and ΔS° appear to be (numerically) smaller in the presence of the divalent cation Ca^{2+}.

Turning specifically to the relevance of these measurements to the processes by which ions stabilize or destabilize macromolecular conformations, it seems to us that the values of $K_{a,rel}$ measured on polyacrylamide are exactly the parameters needed to calculate the *difference* between the free energy of transfer of a peptide unit plus an average "internal" amino acid side chain from the "interior" of a protein into a solution of stabilizing or destabilizing salt, and the free energy for the same transfer from the protein interior into pure water ($\Delta(\Delta G_{tr})_{res}$). That is, these values can be used to calculate the *change* in free energy stabilizing a folded protein relative to the thermally induced unfolded form which accompanies the transfer of the protein to a different solvent environment. Such calculations may be made using the following equation (Schellman, 1955)

$$\Delta G_{tr,salt\ solution} - \Delta G_{tr,H_2O} = \Delta(\Delta G_{tr})_{res} = -RT\sum_i \ln (1 + K_{a,rel,i}[C_i]) \quad (3)$$

where $\Delta G_{tr,salt\ solution}$ and $\Delta G_{tr,H_2O}$ represent the change in the free energy of the acrylamide unit in going *from* an unsolved environment *to* salt solution or water respectively, $[C_i]$ represents the concentration of ionic species i, and $K_{a,rel,i}$ represents the relative equilibrium constant for salt binding to the amide moiety in polyacrylamide, as defined by eq 2. Representative values of such single ion parameters (for 1 M concentrations of ions) are tabulated in Table III.[7] Such values can be calculated for any solution of neutral salts using eq 3 and the concept of the additivity of ion effects, and provides an estimate of $\Delta(\Delta G_{tr})$ per average peptide unit exposed to the ionic solvent in the process of macromolecular unfolding (denaturation) or dissociation. The value of $\Delta(\Delta G_{tr})$ per molecule can then be calculated directly for any transition in which the number of peptide groups exposed in a particular unfolding process is known. Alternatively, knowing $\Delta(\Delta G_{tr})$ per residue for transfer into the particular salt solution being used, one can use the well-known melting temperature depression equation (*e.g.*, see von Hippel and Schleich, 1969a)

$$T_m - T_m^\circ = n[R(T_m^\circ)^2/\Delta H_m^\circ]\sum_i \ln (1 + K_{a,rel,i}[C_i]) \quad (4)$$

where T_m and T_m° are the melting temperature of the macromolecule in the denaturing (or stabilizing) neutral salt solution and in water (or standard buffer), respectively, and ΔH_m° is the standard enthalpy change for the macromolecular unfolding process into the solvent under consideration. This equation may be used to *calculate n*, the number of amide moieties exposed in the unfolding transition. The appropriateness of the acrylamide data as a model for the actual polar to nonpolar ratio of the groups exposed in any particular unfolding or dissociation process can be checked by demonstrating that the transition from stabilizing to destabilizing effects for that macromolecular process indeed falls near NaCl in the ion effectiveness ranking (*e.g.*, Table I). This approach will be applied to actual protein systems in subsequent papers (*e.g.*, Talbot and von Hippel, manuscript in preparation).

In this paper we have provided additional direct evidence that the specificity of the effects of neutral salts on the stability of macromolecular conformations lies in the binding to the amide group. How this specificity is controlled by the nature and distribution of nonpolar groups *around* the amide dipole itself forms the basis of the next paper (Hamabata and von Hippel, 1973).

[7] Since $K_{a,rel}[C]$ is generally $\ll 1$, the logarithmic term can generally be replaced by $K_{a,rel}[C]$ itself in these calculations.

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Model Studies on the Effects of Neutral Salts on the Conformational Stability of Biological Macromolecules. II. Effects of Vicinal Hydrophobic Groups on the Specificity of Binding of Ions to Amide Groups†

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ABSTRACT: A series of measurements of the affinity of several neutral salts (NaCl, NaBr, NaI, and $NaClO_4$) for water, relative to their affinity for variously substituted small molecule amides, is reported. The measurements were made by binding the salts, as ion pairs, to ion-retardation resins from which the salts can be displaced by water and other polar, but uncharged, eluents. The relative affinity of water and the amides for the salts, as well as the specificity of salt binding to variously substituted amide dipoles, has been determined. It is shown that the affinity of the amides for salt decreases as increasing numbers of methyl substituents are placed around the amide dipole in the order: formamide > acetamide $\simeq$ *N*-methylformamide > *N*-methylacetamide $\simeq$ *N*,*N*-dimethylformamide > *N*,*N*-dimethylacetamide. Thus, the affinity of the amide dipole for neutral salts decreases approximately in proportion to the degree of methyl substitution. However, it is also shown that methyls substituted at different sites have quantitatively different effects on ion binding to the amide dipole. We find that the mono-monovalent salts tested all bind approximately equally to an "ideal" amide dipole resembling, but not identical with, formamide, and that the measured affinities diverge for the various salts as methyl groups are added. The affinity decreases most rapidly per added methyl group for NaCl, and progressively less precipitously for NaBr, NaI, and $NaClO_4$, in that order. Thus, the Hofmeister specificity of neutral salt binding to amides appears to originate in the "modulation" of the binding by the vicinal nonpolar groups. The quantitative results are compared with thermodynamic data obtained by others by solubility measurements, and possible molecular origins of the observed effects are discussed.

In the preceding paper (von Hippel *et al.*, 1973) it was shown that neutral salts bind to the amide groups of polyacrylamide gels with relative (to water) binding constants ($K_{a,rel}$) that follow the rankings of the Hofmeister series in terms of the effects of these ions on the conformational stability of biological macromolecules. In direct proportion to the effect on macromolecular order $\rightleftarrows$ disorder transition temperatures, destabilizing ions (*e.g.*, ClO_4^-, I^-, SCN^-, Ca^{2+}) show positive values of $K_{a,rel}$ for binding to polyacrylamide and "inert" ions (*e.g.*, Cl^-, Na^+, K^+) exhibit relative binding constants close to zero, while ions which tend to stabilize macromolecular conformations (*e.g.*, SO_4^{2-}, F^-) are characterized by negative values of $K_{a,rel}$. Thus, both in terms of the order and the sign of the relative binding constants, the ion-bonding properties of the acrylamide moiety serve as a good model for the behavior of a peptide group and the associated average side chain exposed to solvent as a consequence of protein unfolding or denaturation.

That these measured values of $K_{a,rel}$ represent "real" (relative) binding constants rather than nonspecific activity coefficient effects (*i.e.*, positive or negative ion-exclusion effects due to differences in water structure around the nonpolar groups of the resin) was demonstrated by showing that neutral salts exhibit no preferential binding (positive or negative) to a totally nonpolar polystyrene matrix, relative to a tritiated water tracer. Thus the actual binding site is the amide dipole, and, as shown in the preceding paper, ion effects on the stability of macromolecular conformations can be calculated in terms of the relevant values of $K_{a,rel}$.

However, these results leave unresolved the sources of the specificity of the ion–amide interactions manifested in the Hofmeister series. One possibility that is the specificity is built into the interaction of the ions with the amide dipole itself, regardless of the attached vicinal groups. The other possibility (which is an extension of the proposal of Schrier and Schrier (1967) based on solubility data) is that ion binding to the amide dipole itself is nonspecific for ions of a given charge type, but becomes specific as a consequence of "modulation" of the binding induced by the vicinal hydrophobic groups.

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