view of these uncertainties and the gentleness of the slopes in Figure 2 one might question the reality of the apparent maximum. The maximum is still apparent, however, if one selects only points with RCH_2NO_2 (R = H, Me, Ar, CH_2=CH) in order to eliminate changes in type of substrate and to minimize steric effects (points 5, 9, 10, 20, 21–26, 31, 33, and 40).

Even though the maximum appears real, it seems clear that the shape of the curve is relatively flat, and that either k^H/k^D is relatively insensitive to the symmetry of the transition state or that the symmetry does not change over wide ranges of ΔpK. The same conclusion is reached from an examination of the relationship of solvent isotope effects and transition state structures in deprotonation reactions,[10] and with respect to the relationship of Brønsted β coefficients to the symmetry of transition states for deprotonation reactions.[11] Expanding on Hammett's statement concerning solvent isotope effects,[12] we conclude that *the hope, which at one time seemed bright, for a simple general correlation of Brønsted coefficients, kinetic isotope effects, and solvent isotope effects with the extent of proton transfer in the transition state has proved vain.*

Acknowledgment. This work was supported by the National Science Foundation (GP 11384).

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Hydration of Macromolecules. III. Hydration of Polypeptides

Sir:

We have described a method for determination of the hydration of proteins.[1] We now report results for water-soluble polypeptides. In this communication we focus on the question: can the hydration of a globular protein be described in terms of the hydration of its constituent amino acids?

Our experiment measures the amount of water which does not freeze when an aqueous macromolecular solution is rapidly frozen and then equilibrated at −20 to −60°. The high-resolution proton nmr spectrum of such a solution shows a single, broad (0.2–2 kHz), lorenztian signal whose area is a direct indication of the amount of unfrozen water. Experimental details and a discussion of the rationale of ascribing this water to "water of hydration" are available.[1,2]

Polypeptides were obtained from commercial sources and used without further purification.[3] All solutions contained 0.01 *M* KCl as well as sufficient KOH or HCl to set the pH as desired. Macromolecular concentrations were 5–10% by weight where possible; otherwise saturated solutions were used. We measured the concentrations of the polypeptides by evaporating a known quantity of solution to dryness (60° for 36 hr, followed by 12 hr at 100°). Absolute hydrations of the polypeptides were obtained by comparing the area of the nmr signal each produced with that found with a 10% bovine albumin sample. The hydration of the albumin had been determined earlier using a D_2O–H_2O–LiCl solution.[1,2]

Our results are given in Table I, with hydration expressed as moles of water per mole of amino acid. The indicated errors include the uncertainties in area and concentration determinations. We find that all the amino acids with ionized side chains are heavily hydrated. In addition, polyproline and the uncharged forms of the basic amino acids hold considerable water. Nonpolar groups hold much less. The nmr water signal increased linearly with polymer concentration (1–10% range), so that the hydration per mole is independent of polymer concentration. No molecular weight dependence was observed. The data indicate a marked temperature dependence for the amount of water associated with the charged residues; a very strong pH effect is seen for the negatively charged glutamic, aspartic, and tyrosyl side chains. We note in passing that the globular proteins, nucleic acids, and nonpolar polypeptides do not show pronounced temperature effects in this temperature range.[1,2]

Four sources of systematic error should be considered. First, we have made no correction for the possible presence of "exchangeable" hydrogens contributing to the water signal. Although many of the polypeptide protons are "readily" exchangeable at room temperature when assayed by conventional methods,[4] it is very unlikely that they are mobile enough to exchange rapidly (by nmr standards) at −30°.[5] Second, we are reasonably confident that there are no contributions to the nmr signal from the CH_2 and CH_3 groups of the side chains. At relatively high temperatures (−10°) the water line is sharp enough so that the CH regions of the spectra are exposed. Under these conditions, only quite small amounts of side-chain resonances are detected, and on lowering the temperature, these quickly broaden and lose intensity as the side chains become immobilized in the ice. Polyvaline is the only exception so far noted. An unusually sharp water peak and a clearly defined CH_3 peak were detectable down to −30°.[6] We cannot report at this time ionic strength and counterion effects. Protein work[1,7] suggests that such effects will

(1) I. D. Kuntz, T. S. Brassfield, G. Law, and G. Purcell, *Science*, **163**, 1329 (1969).
(2) I. D. Kuntz and T. S. Brassfield, *Arch. Biochem. Biophys.*, in press.
(3) The source, molecular weight as supplied by the manufacturer, and concentration used in the nmr experiments (wt %) are given for each polypeptide: DL-Ala, S, 1600, 4%; L-ArgHCl, S, 50,000, 5%; L-Asn, P, mol wt unknown, 5–9%; Gly, S, 10,000, 0.35%; L-LysHBr, M, 12,000 and 44,000, 2–9%; DL-LysHBr, M, 60,000, 7%; L-OrnHBr, P, 105,000, 6–10%; L-Pro, S, 7500 and 200,000, 2 and 3.5%, respectively; L-Tyr, P, 100,000, 3%; L-Val, M, mol wt unknown, 0.4%; $Lys^{40}Glu^{60}$, from W. Kauzmann who received it from E. Blout, mol wt unknown, 5–9%; $Lys^{50}Phe^{50}$, M, 180,000, 5%; $Lys^{33}Phe,^{67}$ M, mol wt unknown, 1.5%. Abbreviations used are: S, Sigma (St. Louis, Mo.); P, Pilot Division of New England Nuclear (Boston, Mass.); M, Miles (Elkhart, Ind.); Sch, Schwartz Biochem. (Orangeburg, N. Y.).
(4) W. Englander in "Poly-α-Amino Acids," G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., Chapter 8.
(5) A. Hvidt and S. O. Nielsen, *Advan. Protein Chem.*, **21**, 287 (1966); A. Wishnia and M. Saunders, *J. Amer. Chem. Soc.*, **84**, 4235 (1962).
(6) Further discussion of the line widths of the nmr signals is given in I. D. Kuntz, *J. Amer. Chem. Soc.*, **93**, 516 (1971).
(7) I. D. Kuntz and R. P. Taylor, unpublished results.

Table I. Polypeptide Hydration

Polypeptide	pH	Hydration[a] −25°	Hydration[a] −35°	Hydration[a] −45°	Error[a]	Conformation[b]
L-Glu	7–12	8.3	7.7	6.3	±1	Coil
L-Glu	4.5		1.8		±0.5	Helix[c]
L-Asp	8–12	8.1	6.0	4.8	±1	Coil
L-Asp	4.5	2.1			±0.5	Helix[c]
L-Tyr	11.5–12		8.5	6.5	±1.5	Coil
L-Tyr	11.3		5.5	5.1	±1	Helix[c]
DL- or L-Lys	3–9	5.0	4.3	3.8	±1	Coil
DL- or L-Lys	10–12	5.0	4.5	3.7	±1	Helix[c,d]
L-Orn	1.5–9	4.0	3.4	3.5	±1	Coil
L-Orn	10–12	4.5	3.7	3.5	±1	Helix[c]
L-Arg	3–8	3.1	2.7		±1	Coil
L-Arg	10	3			±1	Helix[c,f]
L-Pro		3.1	2.8		±1	*e*
L-Asn		2.0			±0.5	?
DL-Ala		1.4			±0.5	Helix[d]
L-Val		0.9			±0.5	Coil,[b] Helix[g]
Gly		0.9			±0.5	?
Copolymers						
$Lys^{40}Glu^{60}$	2–4	2.5	2.4		±0.5	Helix
$Lys^{40}Glu^{60}$	11–12	7.8	7.5		±1	Coil
$Lys^{50}Phe^{50}$	2–9	2.6	3.8		±0.5	*f*, *h*
$Lys^{33}Phe^{67}$		1.2			±0.5	?

[a] As moles of water per mole of amino acid. Includes estimates of error in area and concentration measurements. [b] Unless otherwise indicated, these assignments are made from literature data.[8] [c] Solubility is very low at extremes of pH. Probably some mixture of helix and coil exists at the pH shown. [d] DL polymers probably contain considerable sequences of D and L residues, permitting helix formation under appropriate conditions.[8] [e] Polyproline II. [f] We assume that this polymer is similar to polylysine. [g] R. Epand and H. Scheraga, *Biopolymers*, 6, 1551 (1968). [h] E. Peggion, A. S. Verdini, A. Cosani, and E. Scoffone, *Macromolecules*, 3, 194 (1970).

be detectable but will be on the order of 20%. Lastly, we have not corrected for end group effects. These should only be important for our sample of poly-DL-alanine.

One of our early concerns was whether water was preferentially bound (or excluded) from particular tertiary structures. Polypeptide solutions are a rich source of conformations including such structures as the α helix, the β-pleated sheet, and extended "random" coils.[8] We have considerable evidence that the line width of the nmr water signal is quite sensitive to conformation effects in these systems.[2,6] But we see little indication of pronounced changes in hydration which can be attributed solely to changes in the polypeptide conformation. For example, both the acidic and basic polypeptides undergo a transition from a random coil charged form to an α-helix structure when uncharged.[8] The hydration of the acidic materials decreases sharply while that of the basic compounds remains unchanged. Thus, while we cannot rule out changes of one water per residue, it seems quite unlikely that, for example, all α helices inherently trap excess water of hydration. Such an effect does seem to occur for the larger double helix structures formed by nucleic acids.[1,9]

To extend our results to a prediction of the hydration of proteins, we must assign a hydration to each of the naturally occurring amino acids, as well as know the composition of the protein and something of its tertiary structure. Table II presents the amino acid hydration assignments. Those residues which we could not measure were assigned values by comparison with similar materials. We have specified a temperature (−35°) and a pH range (6–8) to permit uniform comparisons. Although many amino acid

(8) See ref 4, Chapter 11.
(9) D. Hendly, A. Broudy, G. Payne, I. D. Kuntz, and J. F. Fresco, in preparation.

Table II. Proposed Amino Acid Hydrations at −35° and pH 6–8 Unless Otherwise Indicated

Amino acid[a]	Hydration[b]	Basis of assignment[c]
	Acidic Groups	
Asp^-	6.0	M
Glu^-	7.5	M
Tyr (uncharged)	3	E
Asp (pH 4)	2	E
Glu (pH 4)	2	M,E[d]
Tyr^- (pH 12)	7.5	M
	Basic Groups	
Arg^-	3.0	M
His^+	4	As Lys^+
Lys^+	4.5	M
Arg (pH 10)	3	M
Lys (pH 10–11)	4.5	M
	Hydrophilic Groups	
Asn	2.0	M
Gln	2	As Asn
Pro-OH	4	*f*
Pro	3.0	M
Ser, Thr	2	*e*
Trp	2	*g*
	Hydrophobic Groups	
Ala	1.5	M
Cys, Met	1	As Val
Gly	1	M
Ile, Leu	1	As Val
Phe	0	M[h]
Val	1	M

[a] Abbreviations as before; arranged by functional group, alphabetically within group. [b] Moles of water per mole of amino acid. [c] M, measured, E, extrapolated from portion of titration curve. [d] Measured from Lys-Glu copolymer assuming Lys value as 4.5. [e] Assuming 1 water per peptide, 1 water per hydroxyl. [f] As Pro plus 1 water per hydroxyl. [g] Assuming 1 water per peptide, 1 water per ring nitrogen. [h] Measured using Lys-Phe copolymers, range of values −0.5 to +0.5.

Table III. Protein Hydration[a]

Protein	State[b]	Model[c]	Calcd[d]	Obsd[e]	Ref
Gelatin	N	FE	0.50	0.45	*l*
Myoglobin	N	FE	0.45	0.415	*f*
Bovine albumin	N	FE	0.445	0.40	*f, l, m*
Bovine albumin	D, urea	FE	0.445	0.44	*m*
Bovine albumin	D, pH 3	FE, T	0.32	0.30	*l*
Hemoglobin	N	FE	0.415	0.42	*l*
Chymotrypsinogen	N	FE	0.39	0.34	*f*
Lysozyme	N	FE	0.36	0.34	*f, l*
Lysozyme	N	FE-B	0.335	0.34	*f, l*
Ovalbumin	N	FE	0.37	0.33	*f*
TMV coat	Intact virus	FE[g]	0.365	0.25	*h*
TMV coat	Stripped virus, pH 10	FE[g]	0.365	0.36[i]	*h*
Chymotrypsin	N	FE	0.36	0.33	*f*
Protamine sulfate	N	FE[j]	0.28	0.28	*f*
1-Lysine, 1-glutamate copolymer	pH 4	FE	3.0[k]	2.4[k]	
	pH 11	FE	6.3[k]	7.5[k]	

[a] As grams of water per gram of protein. [b] N, native protein, 0.01 *M* KCl; D, denatured protein, as shown. [c] FE, all residues fully exposed; FE, T, residues fully exposed but all carboxylate ions assumed titrated; FE-B, corrected for buried residues. [d] Residue hydration taken from Table II. Amino acid analyses from ref 10 and 11. [e] Results from ref 1 have been revised slightly to yield bovine albumin hydration of 0.40, in agreement with later work. [f] This work, proteins run as described in ref 1, and 2. [g] RNA is assumed to be unhydrated. [h] W. Bostian and I. D. Kuntz, unpublished results. [i] Corrected for RNA hydration assumed to be 0.07 g of water per gram of intact virus. [j] Corrected for weight of sulfate as counterion. [k] As moles of water per mole of amino acid. [l] See ref 1. [m] See ref 2.

compositions are available,[10,11] few explicit assignments of "protected" and "exposed" residues have been made.[12] Thus, we have assumed that each amino acid is exposed to the solvent. This should lead to an overestimate of the hydration. The magnitude of this overestimate can be roughly evaluated using lysozyme where the protected residues have been identified.[12] Alternatively, one could measure the hydration of completely denatured proteins which should approximate the "fully" exposed state.[13] We have ignored all contributions from other "structural" features, such as grooves, channels, helical regions, etc.

Calculated and experimental (nmr) results are given in Table III. Remember that the absolute hydration of both the polypeptides and proteins has been based on the assumption that bovine albumin has a hydration of 0.40 ± 0.04 g of water/g of protein. The error in the protein measurements is on the order of 10%. The calculated values appear consistently high although within the experimental error. The correction for buried residues is about 10% in the case of lysozyme. This correction should increase as the size of the (globular) protein increases. It is encouraging that both the urea and acid-denatured samples of serum albumin agree with the simple predictions: *i.e.*, the urea exposes the hydrophobic groups which pick up only a small amount of water; acid, by titrating the carboxyl groups, dehydrates the protein. The only striking "structural" effect in Table III is a negative one. Tobacco mosaic virus is much less hydrated than the calculations indicate. When the coat protein is dissociated, the hydration increases to the expected value. The comparison of hemoglobin and myoglobin suggests that aggregation of subunits, *per se*, does not generate a major change in hydration (see also ref 14). We rationalize this finding using the "hydrophobic binding" model for subunit association. If only poorly hydrated hydrophobic regions are involved, one expects small (<10%) decreases in water binding. Conversely, the protein–RNA interface in TMV must contain a large percentage of ionic groups to cause such a precipitous decrease in hydration.

In conclusion, the nmr determination of hydration yields an internally consistent method of estimating the hydration of globular proteins in terms of the hydration of the appropriate polypeptides. The major part of the water is associated with the ionic side chains, although 25–30% is "bound" by the nonionic groups. We predict that the hydration of proteins should be quite sensitive to acid titration but should be insensitive to pH above pH 6 (assuming no major structural changes). Fully exposing the hydrophobic core of globular proteins should produce a small increase in hydration. Aggregation which involves hydrophobic sites should slightly decrease hydration, while aggregation which protects ionic sites should cause large decreases.

Further work is in progress on several of these topics.

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(12) I. Klotz, *Arch. Biochem. Biophys.*, **138**, 704 (1970), and references therein.
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Hydration of Macromolecules. IV. Polypeptide Conformation in Frozen Solutions

Sir:

In a companion paper we have discussed the use of an nmr method of determining the hydration of water-soluble polypeptides.[1] In this communication we show that the nmr technique is sensitive to the polypeptide conformation, even though the hydration, *per se*, appears independent of the structure of the polypeptide.[1]

(1) I. D. Kuntz, *J. Amer. Chem. Soc.*, **93**, 514 (1971).