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Preferential Binding of Solvent Components to Proteins in Mixed Water–Organic Solvent Systems*

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ABSTRACT: The preferential interaction of lysozyme, bovine serum albumin, and insulin with one of the solvent components in water–2-chloroethanol mixtures has been investigated by the method of differential refractometry with the application of multicomponent theory. Similarly to the case found with β-lactoglobulin, as the chloroethanol contents increase, the three proteins interact preferentially first with 2-chloroethanol; then, after passing a maximum between 30 and 40 vol % of chloroethanol, this interaction decreases and is followed by a change to preferential hydration at about 60 vol %.

The preferential interaction of β-lactoglobulin A with solvent components in mixtures of water with ethylene glycol and methoxyethanol has been studied with the same technique. In these solvents, the effect is weaker than in the 2-chloroethanol system. In both systems, no significant excess binding of solvent components is detected below 30 vol %. Above this solvent composition, the organic solvent becomes progressively preferentially bound. These results are compared with those of conformational transition studies carried out in the same systems, and they are discussed in terms of the affinities of different amino acid residues for various types of media as the protein conformation is altered by the change in the medium.

When a macromolecule is dissolved in a mixed solvent (*e.g.*, water–single electrolyte or water–organic solvent), in general it will have a greater affinity for one of the solvent components and, therefore, will interact preferentially with that component over the other one. Such a preferential interaction can be detected by a variety of methods, for example, by a measurement of the buoyant behavior of the macromolecule in a density gradient (Cox and Schumaker, 1961; Vinograd and Hearst, 1962; Ifft and Vinograd, 1966), by the isopiestic measurement of vapor pressure (Hade and Tanford, 1967), by equilibrium sedimentation experiments in solvents of different densities (Schachman and Edelstein, 1966), by light-scattering measurements (Ewart *et al.*, 1946; Kay and Edsall, 1956; Read, 1960; Stauff and Mehrotra, 1961; Inoue and Timasheff, 1968a,b), by the comparison of the partial specific volume or refractive index increment before and after redistribution of solvent components across a membrane impermeable to the macromolecule (Vrij, 1959; Kielley and Harrington, 1960; Casassa and Eisenberg, 1961; Vrij and Overbeek, 1962; Noelken and Timasheff, 1967; Inoue and Timasheff, 1968b). The last method is rather simple to use if the two components of the mixed solvent have nonidentical refractive indices.

Partial specific volume (Kielley and Harrington, 1960), isopiestic (Hade and Tanford, 1967), and refractive index increment studies (Noelken and Timasheff, 1967) have shown that many proteins bind preferentially the salt component when dissolved in aqueous guanidine hydrochloride, if the reference state is chosen as that of equal molality of the salt on the two sides of the membrane. Light-scattering and refractive index increment measurements (Stauff and Mehrotra, 1961; Inoue and

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Timasheff, 1968b) have shown that the organic solvent, 2-chloroethanol, interacts with bovine serum albumin and β-lactoglobulin A in a more complicated manner. At low alcohol concentration, these proteins interact preferentially with 2-chloroethanol, becoming preferentially hydrated at high concentrations of the organic component. While guanidine hydrochloride disrupts ordered protein structures, 2-chloroethanol is considered as a protein structure enhancing agent (Doty, 1959; Callaghan and Martin, 1962). In the case of β-Lg A,[1] for example, the conformation is changed from the native structure to one greatly enriched in α helix as the concentration of 2-chloroethanol changes from 10 to 20% by volume (Inoue and Timasheff, 1968b). It seemed of interest, therefore, to examine the relation between the helix-inducing ability of some organic solvents and the pattern of their preferential interaction with proteins in an aqueous medium. For this purpose, the systems water–ethylene glycol and water–methoxyethanol were investigated, using β-lactoglobulin as protein. Also, the behavior of 2-chloroethanol was further examined by extending the study of its interactions to the proteins lysozyme, insulin, and BSA. These studies were carried out using principally the differential refractometric method. It is the purpose of this paper to report the results.

Theory

In treating a three-component system, we have chosen the notation of Scatchard (1946) and Stockmayer (1950), in which the macromolecular solute is component 2, water is component 1, and the organic solvent is component 3. Expressing the concentration on the molal scale, the preferential binding[2] of component 3 to the macromolecule is measured directly by the difference between the refractive index increments measured at conditions at which the chemical potential of the third component and its molality are, in turn, kept identical in the solution and in the solvent. In eq 1, n is the

$$\left[\frac{\partial n}{\partial m_2}\right]_{T,p,\mu_3} = \left[\frac{\partial n}{\partial m_2}\right]_{T,p,m_3} + \left[\frac{\partial n}{\partial m_3}\right]_{T,p,m_2}\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} \quad (1)$$

refractive index, m_i is the molal concentration of component i (moles/1000 g of the principal solvent, or component 1), μ_i is its chemical potential, T is the thermodynamic temperature, and p is the pressure. In practice, it is more convenient to measure the concentration in grams per milliliter of solution; changing to these units of concentration, and extrapolating the concentration of component 2 to zero, we obtain

$$\frac{M_3}{M_2}\left[\frac{\partial m_3}{\partial m_2}\right]^0_{T,p,\mu_3} = \left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,p,\mu_3} = \frac{1}{(1-\bar{V}_3C_3)}\left\{\left[\frac{\partial n}{\partial C_2}\right]_{T,p,\mu_3} - \left[\frac{\partial n}{\partial C_2}\right]_{T,p,m_3}\right\}\Big/\left[\frac{\partial n}{\partial C_3}\right]_{T,p,m_2} \quad (2)$$

where M is the molecular weight, g is the concentration in grams per gram of component 1, $\bar{V}$ is the partial specific volume measured in the usual way, and C is the concentration in grams per milliliter of solution; the superscript 0 indicates extrapolation to zero of the protein concentration. The refractive index increment at constant molality of component 3, $(\partial n/\partial C_2)_{T,p,m_3}$, is measured on protein solutions using as reference a solvent having the same molality of component 3; that at constant chemical potential of component 3, $(\partial n/\partial C_2)_{T,p,\mu_3}$, is approximated in practice by $(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$, which is obtained by taking the difference between the refractive index of the protein solution, which had been equilibrated by dialysis with the solvent, and the dialysate. Since the difference between $(\partial n/\partial C_2)_{T,p,\mu_3}$ and $(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$ is, in general, very small (Stigter, 1960), we will use in the following equations the value of $(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$ instead of $(\partial n/\partial C_2)_{T,p,\mu_3}$ (the use of $(\partial n/\partial C_2)_{T,p,\mu_3}$ would require that all measurements be carried out under a hydrostatic head equal to the osmotic pressure of the solution). The amount of preferential binding, $(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ or $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, is defined as zero when the amount of third component per 1000 g, or per g, of water, respectively, is identical on both sides of the membrane at osmotic equilibrium. On the other hand, when we define as zero preferential binding the state in which the amount of third component per milliliter of solution is identical on the two sides of the dialysis membrane, the preferential binding is expressed by $(\partial C_3/\partial C_2)_{T,\mu_1,\mu_3}$, and is given by eq 3, in which the refractive index increments are measured at constant molarity and constant chemical potential of the third component, respectively (Vrij and Overbeek, 1962; Noelken and Timasheff, 1967)

$$\left[\frac{\partial C_3}{\partial C_2}\right]_{T,\mu_1,\mu_3} = \left\{\left[\frac{\partial n}{\partial C_2}\right]_{T,\mu_1,\mu_3} - \left[\frac{\partial n}{\partial C_2}\right]_{T,p,C_3}\right\}\Big/\left[\frac{\partial n}{\partial C_3}\right]_{T,p,C_2} \quad (3)$$

These two types of preferential binding are related by the following equation (Noelken and Timasheff, 1967; Inoue and Timasheff, 1968a)

$$\frac{M_3}{M_2}\left[\frac{\partial m_3}{\partial m_2}\right]_{T,\mu_1,\mu_3} = \left[\frac{\partial g_3}{\partial g_2}\right]_{T,\mu_1,\mu_3} = \frac{(1-C_2\bar{V}_2)_{T,\mu_1,\mu_3}}{(1-C_3\bar{V}_3)_{T,\mu_1,\mu_2}}\left[\frac{\partial C_3}{\partial C_2}\right]_{T,\mu_1,\mu_3} = \left[\frac{\partial C_3}{\partial C_2}\right]_{T,\mu_1,\mu_3} \times \frac{[1-C_2(\bar{V}_2)_{T,p,m_3} - C_2(\bar{V}_3)_{T,p,m_2}(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}]}{[1-C_3(\bar{V}_3)_{T,p,m_2} - C_3(\bar{V}_2)_{T,p,m_3}(\partial g_2/\partial g_3)_{T,\mu_1,\mu_3}]} \quad (4)$$

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: β-Lg A, β-lactoglobulin A; BSA, bovine serum albumin.

[2] The term binding here is taken in its most general thermodynamic sense, namely, it indicates that, within the immediate domain of the macromolecules, there is an excess of a solvent component over the bulk composition of the solvent. No information is given on attachment of solvent to solute at specific sites; such deductions necessarily require the assumption of molecular models.

The use of this equation requires measurements of the partial specific volumes of components 2 and 3 both at constant molality and constant chemical potential of components 3 and 2, respectively; $\bar{V}_2$ measurements must be made with reference to solvents which, in turn, have the same molality of component 3 as is found in the solution and which have been equilibrated by dialysis with component 3. Extrapolating C_2 to zero, eq 4 reduces to

$$\left[\frac{\partial g_3}{\partial g_2}\right]^0_{T,\mu_1,\mu_3} = \frac{g_3}{(\bar{V}_1)_{T,p,m_2}C_3}\left[\left[\frac{\partial C_3}{\partial C_2}\right]^0_{T,\mu_1,\mu_3} + C_3(\bar{V}_2)_{T,p,m_3}\right] \quad (5)$$

Positive values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ or $(\partial C_3/\partial C_2)_{T,\mu_1,\mu_3}$ signify preferential binding of component 3 to the macromolecule; negative values indicate preferential hydration. The values of preferential binding of component 1 to component 2 can be calculated by (Timasheff and Kronman, 1959)

$$\left[\frac{\partial g_1}{\partial g_2}\right]_{T,\mu_1,\mu_3} = -\frac{g_1}{g_3}\left[\frac{\partial g_3}{\partial g_2}\right]_{T,\mu_1,\mu_3}$$
$$\left[\frac{\partial C_1}{\partial C_2}\right]_{T,\mu_1,\mu_3} = -\frac{C_1}{C_3}\left[\frac{\partial C_3}{\partial C_2}\right]_{T,\mu_1,\mu_3} \quad (6)$$

Preferential interaction can be measured also by the light-scattering technique. When the scattered intensities of protein solutions are measured in a water–organic solvent mixture, keeping the molality of the organic solvent identical in the solvent and solution, multicomponent theory results in the equation (Zernicke, 1918; Brinkman and Hermans, 1949; Kirkwood and Goldberg, 1950; Stockmayer, 1950; see also Inoue and Timasheff, 1968b)

$$H'\left[\frac{\partial n}{\partial C_2}\right]^2_{T,p,m_3}\frac{C_2}{\Delta\tau} = \frac{1}{(1+D)^2}\left[\frac{1}{M_2} + 2B^0C_2\right] + 0(C_2^2) \quad (7)$$

$$H' = \frac{32\pi^3n^2}{3N\lambda^4} \quad (7a)$$

$$D = \frac{(\partial n/\partial m_3)_{T,p,m_2}}{(\partial n/\partial m_2)_{T,p,m_3}}\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} = \frac{(1-C_3\bar{V}_3)_{m_2}(\partial n/\partial C_3)_{T,p,m_2}}{(1-C_2\bar{V}_2)_{m_3}(\partial n/\partial C_2)_{T,p,m_3}}\left[\frac{\partial g_3}{\partial g_2}\right]_{T,p,\mu_3} \quad (7b)$$

$$B^0 = \frac{1}{m_2^2}\left[\frac{V}{2}\left\{\beta_{22} - \frac{\beta_{23}^2}{\frac{\Sigma\nu_3}{m_3} + \beta_{33}}\right\} + \bar{V}_2M_2\right] \quad (7c)$$

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3} = -\frac{\beta_{23}}{\frac{\Sigma\nu_3}{m_3} + \beta_{33}} \quad (7d)$$

In this equation, N is Avogadro's number, λ is the wavelength of the light *in vacuo*, V is the volume of solution which contains 1000 g of component 1 (water), $\Sigma\nu_3$ is the number of particles into which component 3 dissociates ($\Sigma\nu_3 = 1$ for an organic solvent), and β_{ij} are interaction constants defined by

$$\beta_{ij} = \frac{1}{RT}\left[\frac{\partial\mu_i^{(e)}}{\partial m_j}\right]_{T,p,m}$$

where $\mu_i^{(e)}$ is the excess chemical potential, $\mu_i = RT\Sigma\nu_i \ln m_i + \mu_i^{(e)} + \mu_i^0(T,p)$. β_{ij} is related to the activity coefficient of i, γ_i, since $\mu_i^{(e)} = RT \ln \gamma_i$.[3] The partial specific volumes in eq 7b and 7c are measured at equal molalities of other components in the solution and reference solvent.

The amount of preferential binding is calculated from the value of D by using eq 7b, if the molecular weight of component 2 is known. Light-scattering measurements keeping the chemical potential of the solvent component identical in solvent and solute (dialysis equilibrium) reduce the three-component equation to a pseudo-two-component equation with the result that extrapolation to zero protein concentration yields the true molecular weight (Ooi, 1958; Vrij, 1959; Stigter, 1960; Casassa and Eisenberg, 1961, 1964; Inoue and Timasheff, 1968a,b).

Experimental Section

Materials. The following proteins were used: β-lactoglobulin A, prepared according to the method of Aschaffenburg and Drewry (1957); lysozyme (salt free), Worthington (lot LYSF 647-8); bovine serum albumin, Pentex (lot 64761); and insulin (bovine pancreatic, Mann lot S3252). All protein solutions used contained 0.02 M NaCl and 0.01 M HCl. The protein concentrations were measured on a Zeiss PMQ II spectrophotometer,[4] using an absorptivity value of 0.96 l./(cm g) at 278 mμ for β-Lg A (Townend *et al.*, 1960), 2.635 l./(cm g) at 281.5 mμ for lysozyme (Sophianopoulos *et al.*, 1962), 0.66 l./(cm g) at 280 mμ for BSA (Tanford and Roberts,

[3] It is by interpreting γ_i in terms of the law of mass action, namely, setting γ_i equal to the fraction of protein which does not interact with component j, that binding constants for ij interactions are obtained. This applies essentially to all measurements of binding by means of thermodynamic techniques. Thus, for the simple stoichiometry

$$P + nS \overset{K}{\rightleftharpoons} PS_n$$

(where P is protein, S is the solvent component, and n is the number of binding sites per protein molecule), the intrinsic binding constant (if all sites are independent), k_{ji}, is given by

$$k_{ji} = [-(\beta_{ij})/nS^{n-1} + S^n\beta_{ij}]^{1/n} \quad (8)$$

For all the interactions studied in the present paper, k_{ji} values can be estimated to be less than unity; thus, the interactions are weak and mass action interpretation would have little meaning.

[4] Mention of companies or products is for the convenience of the reader and does not constitute an endorsement by the U. S. Department of Agriculture.

TABLE I: Densities and Partial Specific Volumes of Components in Mixtures of Water with Ethylene Glycol and with Methoxyethanol at 25.0°.

Org Solv (vol %)	Ethylene Glycol			Methoxyethanol		
	Density (g/ml)	$\bar{V}_1$ (ml/g)	$\bar{V}_3$ (ml/g)	Density (g/ml)	$\bar{V}_1$ (ml/g)	$\bar{V}_3$ (ml/g)
0	0.9971			0.9971		
10	1.0110	1.0029	0.8788	0.9993	1.0033	0.9756
20	1.0251	1.0026	0.8771	1.0022	1.0037	0.9732
30	1.0391	1.0012	0.8801	1.0051	1.0018	0.9775
40	1.0522	0.9992	0.8833	1.0068	0.9967	0.9877
50	1.0647	0.9948	0.8882	1.0061	0.9845	1.0042
60	1.0762	0.9879	0.8930	1.0029	0.9733	1.0149
70	1.0865	0.9820	0.8960	0.9972	0.9591	1.0238
80	1.0957	0.9747	0.8982	0.9888	0.9381	1.0322
90	1.1038	0.9652	0.8998	0.9769	0.9087	1.0383
100	1.1104			0.9605		

1952), and 1.04 l./(cm g) at 277 mμ for insulin (Herskovits, 1965). The values of molecular weights of the proteins used in calculations were 18,100 for β-Lg A (measured by light scattering (Inoue and Timasheff, 1968b)), 14,100 for lysozyme (Smith *et al.*, 1956), 70,000 for BSA (Klainer and Kegeles, 1955), and 12,000 for insulin (Doty and Myers, 1953). Neither $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ nor $(\partial C_3/\partial C_2)_{T,\mu_1,\mu_3}$ are functions of molecular weight. The organic solvents, 2-chloroethanol, ethylene glycol, and methoxyethanol (Eastman Organic Chemicals), were doubly distilled immediately before use. The dialysis tubing was obtained from Union Carbide Co.

Refractive index increments of the proteins were measured on a Brice differential refractometer (Brice and Halwer, 1951) at 436 mμ at 25°. For measurements of $(\partial n/\partial C_2)_{T,\mu_1,\mu_3}$ the two components of the differential cell contained a protein solution which had been brought to dialysis equilibrium with the solvent and its dialysate, respectively. The values of $(\partial n/\partial C_2)_{T,p,m_3}$ were determined as described before (Inoue and Timasheff, 1968b). The crystalline β-Lg A used contained about 7% water by weight. The exact amount of water was determined from the difference between the concentration of β-Lg A obtained by weighing the protein and that obtained from optical density measurements. For the determination of $(\partial n/\partial C_2)_{T,p,C_3}$ the protein solution and its reference were prepared as follows. An aqueous stock solution (1 ml) (*ca.* 20 g/l. in 0.02 M NaCl and 0.01 M HCl) was delivered into a 5-ml volumetric flask by a volumetric pipet; then the predetermined volume of the organic solvent was added to yield a solution of a given C_3, and the flask was filled to the line by adding a mixed solvent of the given C_3. A measurement was made then of the difference between the refractive indices of this solution and the reference solvent which was made up by the same procedure, using aqueous 0.02 M NaCl and 0.01 M HCl instead of the stock protein solution. Both the solution and the solvent were then diluted with solvent of concentration C_3 and the refractive index difference was measured again. The dilution was repeated until the protein concentration had reached 0.5 g/l. These values were then extrapolated to zero protein concentration. In all cases they were found to be independent of protein concentration between 4 and 0.5 g/l. Least squaring of the results gave a probable error in $\partial n/\partial C_2$ values of ±0.002 ml/g.

The refractive index increments of ethylene glycol and methoxyethanol in aqueous solutions $(\partial n/\partial C_3)_{T,p,m_2}$ were obtained by measuring the refractive index at several concentrations with a Bausch and Lomb precision refractometer at 25° at 436 mμ, and drawing the tangents of the plots of the refractive indices *vs.* the concentration of the organic solvent. The refractive increments of 2-chloroethanol have been reported earlier (Inoue and Timasheff, 1968b). All these values were measured in the absence of protein, assuming that addition of protein would have no effect either on $(\partial n/\partial C_3)_{T,p,m_2}$ or $(\partial n/\partial C_3)_{T,p,C_2}$ (Vrij and Overbeek, 1962), especially since all preferential binding results were calculated after extrapolation to zero protein concentration.

The partial specific volumes of the components in water–ethylene glycol and water–methoxyethanol mixtures were determined by the usual method (Lewis and Randall, 1923). The values are listed in Table I together with the densities of these mixtures.

Light-scattering measurements were carried out on the β-Lg A solutions in water–methoxyethanol mixtures at the wavelength of 436 mμ at 25°, using a Brice photometer (Brice *et al.*, 1950). All measurements were done at constant molality of component 3, using the previously described technique.

Equilibrium Sedimentation. Apparent molecular weights of β-Lg A were determined in water–2-chloroethanol mixtures using the Yphantis (1964) meniscus depletion technique with 4-mm solution columns. The experiments were carried out at 25° in a Spinco Model E analytical ultracentrifuge, equipped with interference

TABLE II: Optical Rotatory Dispersion Parameters of β-Lg A in Methoxyethanol.

Methoxyethanol (vol %)	$-a_0$	$-b_0$	$-[m']_{233}$[a]	$[m']_{200}$[a]	App[b] % Helix
0	160	94	1,500 (229)	7,300 (203)	15
30	213	137	2,300	10,100 (202)	22
40	266	202	5,300	18,400	32
60	159	363	8,500	40,000	58
80	70	437	9,100	50,600	69
100	11	467	9,500	51,000	74

[a] The actual wavelengths of peak and trough positions are indicated in parentheses. [b] From b_0, using $b_0 = -630$ for 100% helix, and 0 for unordered and β conformations.

optics, at appropriate speeds calculated by the Yphantis method for solutions of the corresponding densities. The plates were analyzed with the help of a Nikon microcomparator. In all cases, the protein concentration was 0.5 g/l. In all calculations, the β-Lg A partial specific volume was taken as 0.751 (Pedersen, 1936).

Results

The preferential binding to β-Lg A of methoxyethanol, 2-chloroethanol, and ethylene glycol in mixtures with water were measured both on the molal, $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$, and the molar, $(\partial C_3/\partial C_2)_{T,\mu_1,\mu_3}$, scales, by the differential refractometric method. All three solvents are known to induce conformational changes in this protein; the effect of ethylene glycol has been studied by Tanford *et al.* (1962) and Kientz and Bigelow (1966); that of 2-chloroethanol was studied by Tanford *et al.* (1960) and Inoue and Timasheff (1968b). Optical rotatory dispersion measurements were carried out, in the present study, on β-Lg A in the presence of methoxyethanol in 0.01 M HCl. These experiments were performed in the same manner as previously, using a Cary 60 spectropolarimeter (Inoue and Timasheff, 1968b). The results are shown on Table II. In the case of this system, as well, the organic additive has little effect on protein structure until a concentration of 40%, above which the apparent degree of helicity increases, as judged from the progressive increase in the negative values of b_0 and $[m']_{233}$ as well as of $[m']_{199}$ with an increase in methoxyethanol contents.

The refractive indices at 436 mμ of ethylene glycol (1.439), methoxyethanol (1.409), and 2-chloroethanol (1.447) at 25° are sufficiently different from that of water (1.340) to yield significant values of $\partial n/\partial C_3$. The results are presented in Table III where the preferential interactions of solvent components with β-Lg A, calculated using eq 2, 3, and 6, are presented. The results for the water–2-chloroethanol system obtained from experiments keeping the molarity of component 3 identical in solvent and solute are also compared with those of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ reported previously (Inoue and Timasheff, 1968a,b).

Light-scattering experiments keeping the molality of component 3 identical in the solvent and solution were also carried out on β-Lg A in the water–methoxyethanol system. Assuming that β-Lg A remains dissociated to the state of a monomer when methoxyethanol is added to a protein dissolved in 0.01 M HCl and 0.02 M NaCl, an assumption which appears reasonable in the light of results in methanol and 2-chloroethanol (Inoue and Timasheff, 1968b), the preferential binding of solvent components to β-Lg A was calculated and the results are presented in Table IV.

The values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ measured at constant solvent molality both by the refractometric and light-scattering techniques and those calculated from $(\partial C_3/\partial C_2)_{T,\mu_1,\mu_3}$ with eq 5 are presented in Figure 1. The agreement between the three sets of values is reasonable both in the 2-chloroethanol and methoxyethanol systems, although on first inspection there appear to be considerable discrepancies. The deviations could be due to two causes, namely, to errors in the determination of $\partial n/\partial C_2$ under the three conditions used and to a lack of knowledge of the exact values of $\bar{V}_2$ in mixed solvents, needed in eq 5. Most of the discrepancies, however, are found to be within the experimental error of ±0.002 in the measurements of $\partial n/\partial C_2$. Examination of Table III reveals that the differences between $[\partial n/\partial C_2]_{T,\mu_1,\mu_3}$ and $[\partial n/\partial C_2]_{T,p,m_3}$ are never greater than 0.007 in the ethylene glycol and methoxyethanol systems. Thus, a small error in $\partial n/\partial C_2$ measurements leads to large errors in binding values. Furthermore, there may be complications resulting from the use of dialysis in one of the measurements. Light-scattering determinations of the same effects, while much more time consuming, have two definite advantages: (1) the light-scattering measurement of preferential binding involves neither the use of membranes nor the redistribution of any solvent components between solvent and solution either by transport in solution or through the vapor phase; (2) the observed effect in light scattering is much greater than that seen be refractormetry, since in light scattering the deviation of the apparent molecular weight from its true value (see eq 7) is proportional to the square of the refractive index increment. Comparison of the three approaches in the water–2-chloroethanol system, where the effects are largest, shows very good agreement be-

TABLE III: Preferential Interaction of Solvent Components with β-Lactoglobulin A on the Molal and Molar Scales, Determined by Differential Refractometry.

Vol %	$\left[\frac{\partial n}{\partial C_2}\right]_{T,\mu_1,\mu_3}$	$\left[\frac{\partial n}{\partial C_2}\right]_{T,p,m_3}$	$\left[\frac{\partial n}{\partial C_2}\right]_{T,p,C_3}$	$\left[\frac{\partial n}{\partial C_3}\right]_{T,p,m_2}$	$\left[\frac{\partial g_3}{\partial g_2}\right]_{T,\mu_1,\mu_3}$	$\left[\frac{\partial C_3}{\partial C_2}\right]_{T,\mu_1,\mu_3}$	$\left[\frac{\partial C_1}{\partial C_2}\right]_{T,\mu_1,\mu_3}$	$(\phi - \phi') \times 10^2$, Calcd
				A. In Water–Ethylene Glycol				
Ethylene Glycol								
20	0.180	0.179	0.187	0.095	0.013	−0.074	0.266	0.1
40	0.168	0.166	0.182	0.093	0.035	−0.150	0.207	0.2
60	0.150	0.145	0.177	0.088	0.140	−0.315	0.188	0.5
80	0.136	0.129	0.172	0.083	0.419	−0.434	0.102	0.6
				B. In Water–Methoxyethanol				
Methoxyethanol								
20	0.181	0.180	0.186	0.092	0.013	−0.057	0.229	0.0
40	0.167	0.167	0.182	0.084	0.000	−0.191	0.286	0.0
60	0.157	0.149	0.178	0.073	0.265	−0.321	0.214	−0.5
80	0.148	0.143	0.175	0.055	0.438	−0.489	0.438	−0.9
				C. In Water–2-Chloroethanol				
2-Chloroethanol								
5	0.192[a]	0.184[a]	0.188	0.103[a]	0.081[a]	0.039		1.6
10	0.195	0.179	0.186	0.103	0.171	0.087		3.1
20	0.198	0.169	0.184	0.100	0.359	0.140		5.5
30	0.202	0.159	0.182	0.094	0.643	0.212		7.8
40	0.192	0.152	0.180	0.092	0.718	0.131		6.8
50	0.175	0.143	0.178	0.089	0.711	−0.034	0.029	5.2
60	0.150	0.137	0.177	0.088	0.366	−0.307	0.175	2.0
80	0.117	0.131	0.175	0.082	−0.848 (0.183)[b]	−0.717	0.155	−2.0

[a] The values in these columns are taken from Inoue and Timasheff (1968b). [b] The value in parentheses is for $(\partial g_1/\partial g_2)_{T,\mu_1,\mu_3}$.

TABLE IV: Molecular Parameters of β-Lactoglobulin A in Water–Methoxyethanol Mixtures Determined by Light Scattering.

MeO-EtOH (vol %)	$I \times 10^{5}$ [a]	M_{app}/M_w [b]	$\left[\frac{\partial m_3}{\partial m_2}\right]_{\mu_1,\mu_3}$ (mole/mole)	$\left[\frac{\partial g_3}{\partial g_2}\right]_{\mu_1,\mu_3}$ (g/g)	$B^0 \times 10^3$	β_{23}	β_{22}
0	5.53	1.000			1.60		
20	5.42	1.020	5.51	0.023	1.51	−1.77	791
40	5.19	1.066	23.94	0.101	2.03	−2.95	881
60	4.90	1.129	72.61	0.305	1.79	−4.05	787
80	5.07	1.091	130.78	0.550	−1.88	−2.85	96

[a] Intercepts at $C_2 = 0$ of plots of $H'(\partial n/\partial C_2)_{m_3}{}^2 C_2/\Delta\tau$ *vs.* C_2. [b] Ratio of apparent molecular weight, which is equal to the reciprocal of I, to the true molecular weight.

TABLE V: Preferential Binding of Proteins on the Molal Scale in Water–2-Chloroethanol, Determined from the Refractive Index Increment Measurement.

Chloroethanol (vol %)	$\left[\frac{\partial n}{\partial C_2}\right]_{\mu_1,\mu_3}$	$\left[\frac{\partial n}{\partial C_2}\right]_{m_3}$	$\left[\frac{\partial g_3}{\partial g_2}\right]_{\mu_1,\mu_3}$	$\left[\frac{\partial g_1}{\partial g_2}\right]_{\mu_1,\mu_3}$
		A. Lysozyme		
20	0.196	0.168	0.347	
40	0.178	0.154	0.431	
60	0.144	0.145	−0.028	0.016
80	0.123	0.131	−0.485	0.104
		B. BSA		
20	0.207	0.172	0.433	
40	0.917	0.1625	0.619	
60	0.1485	0.1525	−0.113	0.064
80	0.1295	0.1425	−0.787	0.170
		C. Insulin		
20	0.197	0.168	0.359	
40	0.186	0.149	0.664	
60	0.145	0.136	0.253	
80	0.117	0.124	−0.424	0.091

tween binding values measured by light scattering and by differential refractometry at constant molality of component 3. The value of $[\partial g_3/\partial g_2]_{T,\mu_1,\mu_3}$, calculated from refractometry results at constant molarity of component 3, however, deviates upward from the other two measurements above 40% chloroethanol. This might be due to the use of 0.751 for $\bar{V}_2$ throughout the calculations; these values had been determined in aqueous solutions (Pedersen, 1936).

Table III and Figure 1 indicate that the pattern of the preferential binding of 2-chloroethanol to β-Lg A is quite different from that of ethylene glycol and methoxyethanol. While 2-chloroethanol binding passes through a maximum, in the case of the other two solvents there is little, if any, preferential interaction below 30–40 vol %; above this solvent composition, the organic solvent becomes progressively more bound up to the highest composition studied. As a result, it seemed of interest to examine other proteins in the water–2-chloroethanol system to determine whether the observed effect is specific either for 2-chloroethanol or for β-Lg A, or both. The results of refractometric measurements, carried out on a molal basis on lysozyme, BSA, and insulin, are presented in Table V, the values of $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ are plotted *vs.* the chloroethanol concentration in Figure 2. It is evident that an effect similar to that obtained with β-Lg A is found with these proteins, as well.

Using the present data on preferential interaction, the effect which these would have on measurements that require knowledge of the partial specific volume of a protein in multicomponent solvents was ascertained.

TABLE VI: Equilibrium Sedimentation of β-Lactoglobulin A in Water–2-Chloroethanol Mixtures.

Chloroethanol (vol %)	App Mol Wt Light Scattering	$\left[\frac{\partial m_3}{\partial m_2}\right]_{T,p,\mu_3}$	ρ_s	$(\phi - \phi') \times 10^2$	App Mol Wt Sed Equil	
					Calcd	Found
0	18,500		0.997	0	18,500	18,800
5	21,000	26.5 ± 20	1.010	1.6 ± 1.2	19,700	18,100[a]
10	22,000	37.5	1.023	3.1	21,100	20,400
30	31,500	164.0	1.071	7.8	26,100	26,000[b]
40	29,800	165.9	1.092	6.8	26,100	26,000
80	15,900	−145.0	1.165	−2.0	15,100	14,600[c]

[a] Average of three measurements, ±800. [b] Average of two measurements, ±600. [c] The plot of log C *vs.* r^2 displayed an upward curvature at the bottom of the cell; this must reflect the aggregation noticed in light scattering at protein concentrations above 2 g/l. (Inoue and Timasheff, 1968b).

As pointed out by Scatchard (1963), the treatment of sedimentation in a multicomponent system was carried out for the first time by J. W. Gibbs some 80 years ago. More recently, Eisenberg (1962) and Casassa and Eisenberg (1964) have written out the following specific expression relating the apparent partial specific volumes of a macromolecule obtained by comparing the density of the macromolecular solution to that of solvent, ϕ, and dialysate, ϕ', respectively, where ρ_s is the density of

$$\phi = \phi' + \left[\frac{\partial g_3}{\partial g_2}\right]_{T,\mu_1,\mu_3}\left[\frac{1}{\rho_s} - (\bar{V}_3)_{T,p,m_2}\right] \qquad (9)$$

the solution and $(\bar{V}_3)_{T,p,m_2}$ is the partial specific volume of the added solvent component measured in the usual way (eq 9). Values of $\phi - \phi'$ have been calculated for β-Lg A from the density data of Table I and of Inoue and Timasheff (1968b) and the binding results of Table

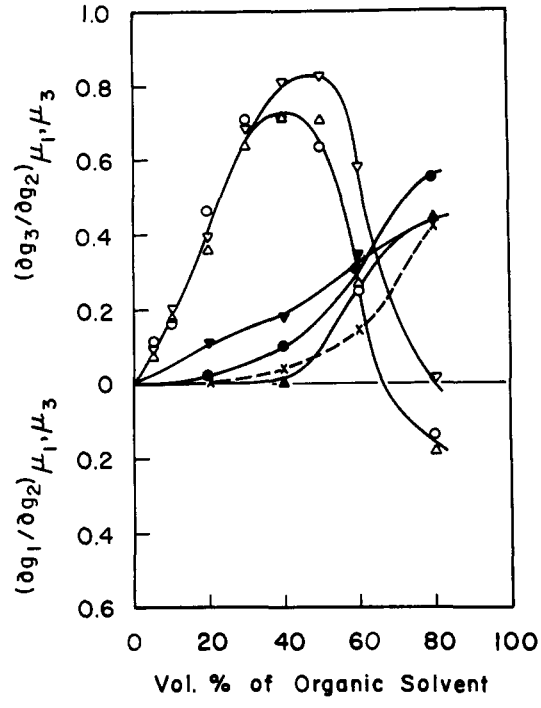

FIGURE 1: Dependence on solvent composition of the preferential interaction of β-lactoglobulin with solvent components. Water–2-chloroethanol system: (○) from light scattering; (△) from $(\partial n/\partial C_2)_{m_3}$ and $(\partial n/\partial C_2)_{\mu_1,\mu_3}$; (▽) from $(\partial n/\partial C_2)_{c_3}$ and $(\partial n/\partial C_2)_{\mu_1,\mu_3}$. Water–methoxyethanol system: (●) from light scattering; (▲) from $(\partial n/\partial C_2)_{m_3}$ and $(\partial n/\partial C_2)_{\mu_1,\mu_3}$; (▼) from $(\partial n/\partial C_2)_{c_3}$ and $(\partial n/\partial C_2)_{\mu_1,\mu_3}$. (×) Water–ethylene glycol system from $(\partial n/\partial C_2)_{m_3}$ and $(\partial n/\partial C_2)_{\mu_1,\mu_3}$.

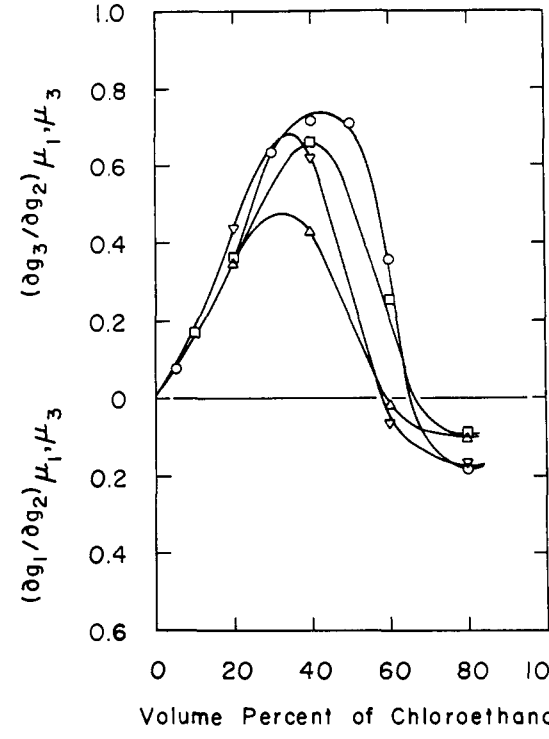

FIGURE 2: Dependence on solvent composition of the preferential interaction of solvent components with various proteins in the water–2-chloroethanol system. (○) β-Lactoglobulin A, (△) lysozyme, (▽) bovine serum albumin, and (□) insulin.

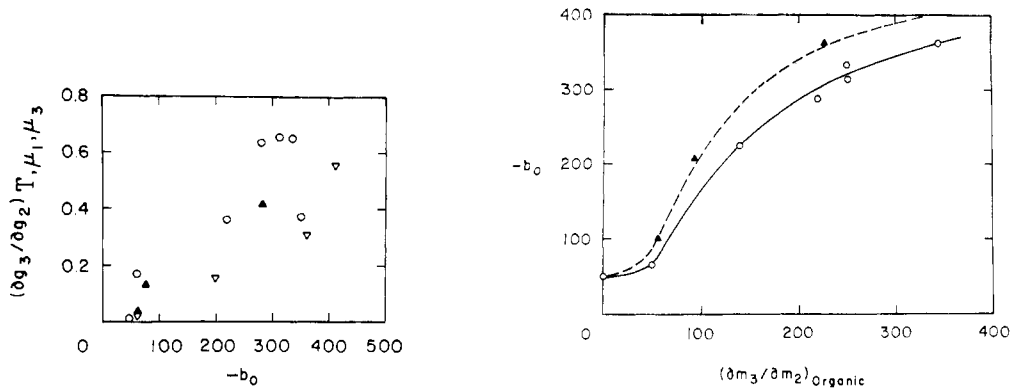

FIGURE 3: Relation between protein conformation and preferential interactions of solvent components with β-lactoglobulin. (A) Left: net binding of organic solvent: (△) methoxyethanol, (▲) glycol, and (○) chloroethanol. (B) Right: binding corrected for strong hydration of polar groups: (○) chloroethanol and (▲) methoxyethanol.

III; these are given in the last column of Table III. In these calculations, the solution density was approximated by that of solvent, a procedure which, at extrapolation to zero concentration, should not introduce any significant errors. It is seen that, in the cases of ethylene glycol and methoxyethanol, neglect of preferential interactions is predicted to result in small errors (not greater than 5%) in the measurement of molecular weights by equilibrium sedimentation; in 2-chloroethanol, however, the expected error would be quite large. The calculated apparent molecular weights in water–2-chloroethanol mixtures, using ϕ rather than ϕ', a relisted in Table VI; the deviations from the true value vary between 41% too high in 30–40% chloroethanol and 20–22% too low in 80% chloroethanol.

Apparent molecular weights in water–2-chloroethanol mixtures were determined by equilibrium sedimentation using $\bar{V}_2 = 0.751$. The results are presented in Table VI. Comparison of these experimental values with those predicted from the light-scattering and refractometric experiments on solvent component binding shows excellent agreement between the two. This result not only emphasizes the magnitude of error inherent in the neglect of multicomponent effects in thermodynamic measurements but also confirms the correctness of the light-scattering and differential refractometry data.

Discussion

The state of aggregation of a protein may be affected by the addition of an organic solvent and a change in the pH of the solution. In the present experiments, all the solutions contained 0.02 M NaCl and 0.01 M HCl; the protein concentrations used were within the range of 5 and 0.5 g/l. Furthermore, since all measurements were extrapolated to zero protein concentration, any tendency of the protein to aggregate should have had a minimal effect on the binding values. Under such conditions the proteins used are known to be in monomeric form, as shown for β-Lg A (Townend *et al.*, 1960; Timasheff and Townend, 1961; Inoue and Timasheff, 1968), lysozyme (Bruzzesi *et al.*, 1965), and insulin (Doty and Myers, 1953). While the differential refractometry method of determining preferential binding is not directly dependent on the state of aggregation of the protein, it is nevertheless preferable to have the macromolecule in a monomeric state, especially if one wishes to use the information in relation with light-scattering or sedimentation equilibrium measurements.

On first inspection, the dependence on solvent composition of the preferential binding of solvent components to proteins appears to follow strikingly different patterns whether one looks at 2-chloroethanol, on one hand, and ethylene glycol and methoxyethanol, on the other hand. The interaction of 2-chloroethanol with four different proteins, however, follows an identical pattern, suggesting that the observed pattern is a property of the solvent and not the protein. All three organic solvents are known to affect protein conformation. 2-Chloroethanol is a strong helix-inducing agent (Doty, 1959; Tanford and De, 1961; Callaghan and Martin, 1962). When this solvent is present in as small amounts as 5–10% by volume, conformational changes are induced which progress with increasing contents of this material (Callaghan and Martin, 1962; Inoue and Timasheff, 1968b).[5] Methoxyethanol and ethylene glycol are increasingly weaker helix-inducing agents. In the case of β-Lg A no structural changes occur at low organic solvent contents; above 30–60% organic component, the apparent helicity of the protein increases.

The relative affinities of the protein for the three solvents, shown in Table III and Figure 1, and the order in which they induce changes in the optical rotatory dispersion parameters of β-Lg A (Tanford and De, 1961; Inoue and Timasheff, 1968b; Table II of this paper) suggest that preferential binding of the organic component and conformational changes may occur in

[5] In the case of insulin, dissolution in pure 2-chloroethanol does not result in any large changes in its optical rotatory dispersion parameters (Doty, 1959), its infrared spectrum (Timasheff *et al.*, 1967), or its circular dichroism spectrum (S. N. Timasheff and L. Stevens, unpublished data). This may, however, be the result of a structure highly constrained by S–S bridges which would impede large conformational changes. Studies now in progress (M. J. Ettinger and S. N. Timasheff, unpublished data) indicate that, as 2-chloroethanol or methoxyethanol contents are increased, insulin undergoes a conformational transition similar to, but smaller than, that undergone by the other proteins.

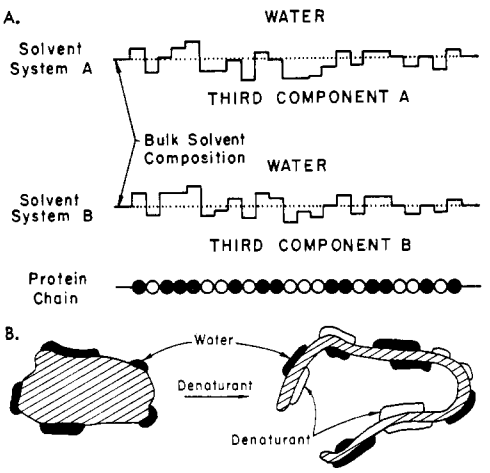

FIGURE 4: Schematic representation of preferential interaction of solvent components with unfolded proteins. (A) Top: solvent composition in the immediate domain of the polypeptide chain, shown for two solvent systems with different affinities for individual amino acid residues; the expected deviations of the solvent composition from average bulk are shown as a function of the degrees of polarity of the amino acid residues; when the composition line is below the bulk solvent composition, the solvent in the immediate vicinity of the protein chain is enriched in water; a composition above the dotted line means enrichment with respect to third component. (○) Polar and (●) nonpolar residue. (B) Bottom: pattern of changes in solvation when protein unfolds; amount of bound water remains essentially unchanged after unfolding, and binding of organic solvent increases.

parallel fashion. In Figure 3A, the binding data for β-Lg A are replotted as a function of the observed b_0 in the various solvents. It is evident that this presentation of the data brings the preferential binding in the three solvents into a more coherent pattern. This suggests that the present observations are of a general character and not specific properties of the particular protein–solvent combinations used. The important factor appears to be the effect which the additive has on protein conformation, *i.e.*, the effect which addition of the third component has on the forces that stabilize the native and altered structures of the proteins, and, therefore, its effect on the free energy of the N ⇌ R reaction, where N and R stand for the native and rearranged forms of the protein. The fact that $(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ for 2-chloroethanol passes through a maximum while it does not do so for the other two solvents is only a reflection of the relative extents of reaction between the protein and the various solvents at given solvent compositions below 80% by volume.

As has been pointed out previously (Inoue and Timasheff, 1968b), in the case of 2-chloroethanol the degree of preferential binding as a function of alcohol contents is not related in any evident form to the shape of the curve representing the N ⇌ R transition under identical conditions (see Figure 5 of Inoue and Timasheff, 1968b). From a knowledge of the free energy of transfer of the various amino acid residues between water and organic solvents (Tanford, 1962; Nozaki and Tanford, 1965), it could be expected *a priori* that as the protein unfolds and its nonpolar residues come into contact with solvent, the organic solvent molecules would tend to cluster about these residues. This should result in preferential binding of the organic solvent as its concentration increases, with a large increase in this binding during the major portion of the transition as more nonpolar residues become exposed and "dissolve" in a layer of the organic solvent. The net effect actually observed, as seen in Figure 1, is considerably more complicated.

A protein contains different types of side chain residue groups, which for the present purpose may be classified simply into polar and nonpolar. As pointed out above, in the mixed solvent, the nonpolar residues have a higher affinity for the organic solvent than for water. The polar residues, on the other hand, are in general located on the surface of the globular molecule in contact with water and are in a hydrated state. As the protein unfolds, each type of residue will continue its preference to interact with water and with the organic additive, respectively. Thus, along the exposed protein chain there will be a nonhomogeneity in affinity toward solvent components leading to a nonhomogeneity in the composition of the medium in immediate contact with the macromolecule. Where polar groups prevail, water will be present preferentially, while the organic solvent will be found in excess along highly nonpolar domains. This situation is depicted schematically in Figure 4A. Looking at each type of residue, in turn, we find that the polar ones are already hydrated in the native form and are thus present in a predominantly aqueous medium prior to unfolding; therefore, as the protein unfolds, few new predominantly water-binding regions are formed; such new regions will consist of the few initially buried polar residues, as well as of the peptide linkages which have a stronger affinity for water than for organic solvents (Nozaki and Tanford, 1965). It is this property of peptide linkages which, according to Nozaki and Tanford (1965), induces strong helix formation in nonhydrogen-bonding organic solvents. Conversely, the nonpolar residues being buried initially and out of contact with solvent will bind increasingly more organic solvent as the protein undergoes the conformational transition. This concept of the total N ⇌ R reaction including changes in solvent binding is depicted schematically in Figure 4B. Taking these considerations into account, the preferential binding of solvent components to protein may be decomposed into two terms; expressing it in terms of preferential binding of water, we have

$$\left(\frac{\partial m_1}{\partial m_2}\right)_{\text{obsd}} = \left[\left(\frac{\partial m_1}{\partial m_2}\right)_{\text{org}} + (A + Bm_3 + Cm_3^2 + \cdots)_{\text{bydr}}\right] \quad (10)$$

The first term on the right-hand side represents the contribution of the preferential binding of the organic solvent to the nonpolar residues as the protein unfolds and is given by eq 5 (this term will be usually negative); the second term represents hydration of the polar groups. The constants *A*, *B*, *C*, etc., represent, respectively, the preferential hydration of the originally exposed polar groups, A, and the dependence of preferential hydration

(positive or negative) on the concentration of the organic solvent, which induces the conformational change.[6] The net observed preferential binding of the organic solvent, $[\partial m_3/\partial m_2]_{obsd}$, then is

$$\left[\frac{\partial m_3}{\partial m_2}\right]_{obsd} = \left[\frac{\partial m_3}{\partial m_2}\right]_{org} - \frac{m_3}{m_1}(A + Bm_3 + Cm_3^2 + \cdots) \quad (11)$$

While values of the constants A, B, C, etc., are not known, sample calculations were carried out keeping only the linear dependence on concentration to see whether this analysis is sensible. In this calculation A was assigned values of 300 and 400 moles of water bound per mole of β-lactoglobulin; this protein has 47 titratable groups plus *ca.* 15 other polar residues per monomeric subunit, and 6–8 waters of hydration per ionizable group seemed reasonable. The value of B was varied between 30 and 60, to indicate a weak concentration dependence (in the case of 2-chloroethanol, m_3 is 1.20 at the highest composition studied). The results of such calculations are presented in Table VII. They show that, when tightly held water of hydration is accounted for specifically, the value of $[\partial m_3/\partial m_2]_{org}$ remains positive at all solvent compositions above the major conformational change. The fact that it is almost constant is consistent with the concept that the initial major change in conformation results in almost total exposure of all residues to solvent (Tanford *et al.*, 1960; Timasheff *et al.*, 1966); the remainder of the transition corresponds to residual folding into α-helical regions. The shape of the experimental binding curve, with negative values of $[\partial m_3/\partial m_2]_{obsd}$ at high 2-chloroethanol concentration, reflects the form of eq 11; thus, when m_3 increases and the water content decreases, the term $(m_3/m_1)(A + Bm_3)$ increases progressively and becomes dominant in the measured preferential binding. Qualitatively this would mean that, as total water content in the system is reduced, the amount of water of hydration tightly held by the ionizable groups, which remains essentially unchanged, results in a solvent composition in the immediate domain of the protein which is richer in water than the bulk solvent, even though the nonpolar residues are dissolved in a local medium progressively richer in organic solvent than the bulk medium. Examination of the calculated $[\partial m_3/\partial m_2]_{org}$ values in terms of the concepts of eq 8 leads to estimates of a binding constant, k, for the organic solvent of somewhat less than unity; such a weak "binding" is too weak to be interpreted in terms of mass action and is certainly insufficient to displace the tightly held water of hydration.

TABLE VII: Calculation of True Binding of 2-Chloroethanol to β-Lg A in Mixtures with Water (A = 400).

Vol %	$(\partial m_3/\partial m_2)_{org}$ B = 30	$(\partial m_3/\partial m_2)_{org}$ B = 60
30	213	220
40	242	250
50	260	260
60	228	240
70	227	245
80	308	345

The indication, noted above, of a correspondence between solvent component binding and b_0 becomes rather striking if we plot now b_0 as a function of $[\partial m_3/\partial m_2]_{org}$, rather than of the net preferential binding, as had been done in Figure 3A. Such a plot is shown in Figure 3B for the 2-chloroethanol system. The monotonic increase of b_0 with $[\partial m_3/\partial m_2]_{org}$ seems to indicate that the conformational change is directly related to the additional solvation of the macromolecule as it unwinds over that found initially with the native protein. A similar relation between the two parameters is obtained as well in the methoxyethanol system, as shown by the dotted line on Figure 3B. That the two sets of points do not fall on a single curve is to be expected, first because the amount of tightly held water may be affected by the nature of the third component, second because the protein unfolding may follow different detailed paths in different solvents, since the various amino acid residues will have different affinities for different solvents; the local solvent compositions that may be expected for two solvent systems composed of water and an organic solvent, with different free energies of transfer of amino acid residues from water to the organic solvent, are depicted schematically in Figure 4A.

In this manner, the apparently anomalous composition dependence of solvent binding is consistent with the concepts (1) that polar residues will tend to be preferentially surrounded by water, while nonpolar ones will be surrounded by nonpolar solvent molecules, and (2) that, in a native protein, the polar residues will be located on the surface, while the nonpolar ones will be principally in the hydrophobic interior of the molecule. The concept of hydration used in the present discussion is the usual one encountered when ionized groups are dissolved in water and it bears no relation to the postulated formation of icebergs proposed for the stabilization of protein structure (Klotz, 1958). Finally, it would appear that in the water–2-chloroethanol system, at least, the ionizable groups remain charged. The evidence is twofold: first, the pure organic solvent has a dielectric constant at 25.8; in mixtures with water, this value should be above that estimated for ion-pair formation (Singer, 1962); second, the protein is in monomeric form in this solvent, and it has been shown that the dissociation at acid pH values is driven by nonspecific electrostatic repulsions (Townend *et al.*, 1960; Timasheff and Townend, 1961).

The pattern of local interactions with solvent com-

[6] The exact form of this concentration dependence is not known; it is expressed here in general form as a virial expansion.

ponents, deduced from the present observations, suggests some further comments on the general mechanism of unfolding of the native globular structure when exposed to organic solvents. The direct correspondence between the conformational change and the predominance of particular solvent components in the immediate domain of specific regions of the protein molecule suggests that the structure of the protein is affected much more strongly by the solvent structure in the immediate vicinity of the molecule, *i.e.*, within the solvation shell, than by that of the bulk solvent. It is generally agreed, at present, that conformational stability of a macromolecule in aqueous medium is the result primarily of the pressure which water exerts on nonpolar residues, forcing these into the interior of the molecule (Kirkwood, 1954; Kauzmann, 1959; Tanford, 1962; Némethy and Scheraga, 1962; Timasheff, 1964; von Hippel and Wong, 1965; Robinson and Jencks, 1965). Conversely, the disruption of a macromolecular structure by the addition of various agents, such as organic solvents, urea, or certain salts, is due to the interaction of these agents with the macromolecule and their effect on the structure of water and the resulting relaxation of the hydrophobic pressure of water on the nonpolar residues. It is, however, not always clear whether it is the bulk water or the first few layers of water about the protein molecule which exert the major effects. In the analysis of this problem for nucleic acid, Sinanoglu and Abdulnur (1964) have come to the conclusion that the deciding solvent property was surface tension and that, consequently, the major role was played by the solvent molecules immediately surrounding the cavity formed by the macromolecular solute. It would seem that the present observation of a direct relationship between changes in the local composition of solvent and relaxation of native protein structure supports this point of view and testifies to the major importance of the first layers of solvent molecules in the immediate vicinity of the macromolecules. The bulk solvent would then be of less immediate importance, although its structure must also be altered both by the addition of the third component and by the particular structure assumed by the solvent in the domain of the unfolding macromolecule and induced by the geometry of the local clusters of polar and nonpolar residues on the protein chain.

The partial sorting out of the solvent molecules in a mixed solvent about the various types of regions present on the protein chain must lead to an unfavorable entropy effect; this, however, may be compensated by the large positive entropy contribution of the unfolding of the protein chain. This concept is supported, furthermore, by the following qualitative considerations. In solubility experiments on amino acids (Cohn and Edsall, 1941; Nozaki and Tanford, 1965) it has been shown that distribution coefficients of polar and nonpolar residues are such that they will tend to redistribute themselves in such manner that the polar ones will be predominantly in the aqueous phase, while the nonpolar ones will be in the organic phase. In a protein these various groups are attached to the polypeptide chain and are, thus, mechanically hindered from migration to the most favorable environment. According to the Le Chatelier principle, a decrease in free energy would be attained in such a case by migration of the small mobile solvent molecules toward the proper residues on the chain, resulting in a local short-range redistribution of solvent components; this could be regarded as an incipient microphase separation on the molecular scale (Nord *et al.*, 1951); conversely this may be looked upon as "dissolution" of individual solvent molecules in regions of different polarities on the protein chain. While it is not possible to propose a detailed kinetic mechanism for this phenomenon in the light of the present-day knowledge of the structures both of proteins and of the used solvents, it may be assumed that the protein and close domain solvent structures become simultaneously altered as a result of contacts and interactions between the various exposed and buried residues and solvent molecules as the protein "breathes," *i.e.*, as it undergoes thermal motions permitting occasional contact of buried residues with solvent. Furthermore, contact between exposed residues on the surface and different types of molecules of a mixed solvent will induce these to alter their mutual orientations in space, contributing to the rapid opening of the inner regions, with resulting cooperative transitions that lead to new structures of the protein and of the solvent both in the solvation layer and in bulk; such new structures would correspond to a new equilibrium state of minimum free energy, within restrictions which may be imposed by kinetic effects, if the structural rearrangements in various parts of the protein molecule occur with different rates. In connection with these remarks, it seems of interest to note that conformational transitions induced by variations in solvent composition are usually a function of a high power of solvent concentration, *i.e.*, they are cooperative (see, for example, Tanford *et al.*, 1960; Tanford and De, 1961; Inoue and Timasheff, 1968b).

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