Inclusion Complexes of Poly-β-cyclodextrin: A Model for Pressure Effects upon Ligand–Protein Complexes†

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ABSTRACT: Certain protein–ligand complexes are destabilized by application of pressures of the order of 5–10 kbar while others are stabilized. This divergent behavior is attributed to differences in compressibility of the protein binding sites. Pressure-stabilized binding is thought by us to be characteristic of "soft binding sites", sites in which rotation about backbone bonds permits reduction of the site dimensions under pressure. In contradistinction, "hard binding sites" do not decrease their size when pressure is applied. As a model for this latter kind we have measured the changes in equilibrium with pressure of complexes of poly-β-cyclodextrin with two fluorescent probes: 8-anilinonaphthalene-1-sulfonate and 6-propionyl-2-(dimethylamino)naphthalene. The standard volume change upon formation of the complexes at 1 atm is similar in both (+9.3 mL/mol), and as expected the incompressibility of the cyclodextrin rings results in a site from which the probes are dissociated by pressure. On the assumption of incompressibility of the binding site, the experimental data permit the calculation of the pressure vs. volume curves (compressibility curves) for the probes molecularly dispersed in water. These curves are in broad agreement with those of liquid aliphatic and aromatic hydrocarbons in the low-pressure range (1–4 kbar) but indicate a reduced compressibility at the higher pressures. Considerations of relative compressibility offer a quantitative alternative to the usual qualitative discussion of the effects of high pressures upon proteins in terms of the participation of "hydrophobic" and other bonds.

High-Pressure Effects upon Protein–Ligand Complexes: The Present State

In recent years a series of observations by several research groups has shown that globular proteins undergo reversible structural transitions when subjected to pressures in the range of 3–11 kbars (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973; Li et al., 1976a,b). Apart from the experiments of Hawley & Mitchell (1975) on the difference in electrophoretic mobility of the low- and high-pressure forms of chymotrypsinogen, these observations refer uniformly to changes of either the absorption spectrum or the fluorescence properties of the aromatic amino acids or of spectroscopically active ligands bound noncovalently to the protein. The aromatic amino acids act effectively as ligands covalently bound at specific points of the protein, and thus the bulk of the observations can be rightly viewed as examples of changes in ligand–protein interactions that take place under pressure. The uniform observation of bathochromic shifts of the fluorescence spectrum of tryptophan in proteins subjected to high pressure (Li et al., 1976a,b; Visser et al., 1977a) indicates that in these cases the internal complexes of this amino acid with other residues of mainly nonpolar character in the protein structure are destabilized at high pressure and replaced by complexes of tryptophan with more polar residues or with water. Similarly, the noncovalent complexes of flavin mononucleotide with the riboflavin binding protein from hen's egg (Li et al., 1976a) and with various flavodoxins (Visser et al., 1977a) or the complex of heme with apometmyoglobin (Zipp & Kauzmann, 1973) are destabilized by pressure. This destabilization is not a general rule for all of the ligand–protein complexes thus far studied. In particular, 8-anilinonaphthalene-1-sulfonate (ANS)[1] is found to bind preferentially (with low stoichiometry, 1:1 and 2:1) to the high-pressure forms of chymotrypsinogen and lysozyme, and this is indirectly inferred to be the case for the binding of triacetylglucosamine by lysozyme (Li et al., 1976b). This diversity of behavior of the ligands can be duplicated in principle by complexes of smaller molecules, where we expect to find similar structural and dynamic features as in the protein complexes, but in a simpler context suitable to an unequivocal analysis.

In agreement with other observers (Kasarda, 1970; Ewald, 1968; Förster et al., 1963) we have found that complexes between two aromatic partners are clearly stabilized by pressure (Weber et al., 1974), but additionally we find that when the two partners are linked by a chain of indifferent methylene groups, a sufficiently short chain operates as a pressure-insensitive link that limits the compressibility of the system and diminishes the standard volume change upon the formation of the complexes (Visser et al., 1977a). This observation agrees with the well-known insensitivity of bond distances and bond angles to pressures (Benson & Drickamer, 1957) and with the very small linear compressibility of diamond (~1% in 100 kbar).

Based on these observations one is led to expect that many, if not all, structural domains of a globular protein molecule will behave as virtually incompressible units unless rotations about some of the covalent bonds permit the decrease in volume of the domain when pressure is applied. One can envision the binding site for FMN in the flavodoxins or the site for the heme in metmyoglobin to be of the kind that does not permit reduction of its size. An "incompressible" binding site of this type will be called a "hard site". On the other hand, when rotation about covalent bonds of the protein backbone permits a decrease in the size of the binding site as pressure is applied, we shall speak of a "soft site". The cleft in the lysozyme molecule that holds the substrates appears to be such a soft site. Application of an external pressure p to a hard site results in a free-energy perturbation of magnitude $p\Delta V$, where ΔV results uniquely from the difference in compressibility between the ligand and the equivalent volume of solute that fills the binding site when the ligand is absent. Both liquid aliphatic and aromatic hydrocarbons are more compressible than water, particularly in the range of 0–5 kbar. On this basis

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[1] Abbreviations used: ANS, 8-anilinonaphthalene-1-sulfonate; PRODAN, 6-propionyl-2-(dimethylamino)naphthalene; PCD, poly-(acroloyl)-β-cyclodextrin.

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we can expect the pressure to have a dissociating effect on such complexes, largely unrelated to the nature of the forces that provide the driving free energy for complex formation at atmospheric pressure. We have searched for relatively simple molecular complexes where the incompressibility of the binding site is evident enough to serve as proof and as a guiding example of the properties of hard binding sites in proteins. The widely studied inclusion complexes of the cyclodextrins (Cramer et al., 1967; Behr & Lehn, 1976; Harada et al., 1977) offer an ideal model system of this type. The covalently bonded ring of sugar residues is not expected to change size when pressures of up to 10 kbar are applied, and the groups in the interior and border of the ring that interact with the included material will maintain their distances at all pressures. This paper describes experiments with the complexes of polymers of β-cyclodextrin (Harada et al., 1976) with two fluorescent probes: 8-anilinonaphthalene-1-sulfonate (ANS) and 6-propionyl-2-(dimethylamino)naphthalene (PRODAN).

Materials and Methods

Magnesium ANS was a recrystallized commercial sample. Its spectral properties are described by Weber & Young (1964) and by Kolb & Weber (1975). The synthesis and the relevant spectroscopic properties of PRODAN are described in the previous paper (Weber & Farris, 1979).

β-Cyclodextrin was obtained from Sigma Chemical Co. and its purity checked by paper chromatography on Whatman No. 1 paper (Wiendenhof, 1964). Poly(acroloyl)-β-cyclodextrin (PCD) was synthesized as described by Harada et al. (1976). Since no compound used in these studies has an ionizable group in the pK range of 2–12, buffering of the solutions was unnecessary. The solvent, therefore, was deionized water further purified by a Millipore Corp. water purification system.

Fluorescence spectra and yield were determined on a photon-counting spectrofluorometer equipped with a pressure cell capable of pressures of 11 kbar (Li et al., 1976a). For all intensity measurements, the absorbancy of the solutions was <0.08 and intensities were corrected for the compression of the solvent. The technical spectra obtained were corrected for instrument response and analyzed using a program capable of fitting up to three simultaneous skewed Gaussians. Lifetimes as a function of pressure were determined by both the phase-shift and modulation methods as described by Spencer & Weber (1969). Mounting the pressure cell in the lifetime instrument necessitated placing the reference scattering solution in an alternate light bypass system, as done by Lakowicz & Weber (1973). To obtain the high light levels necessary for lifetime measurements, the absorbancy of the ANS was 0.4. Excitation was at 365 nm, and emission was viewed through a Corning 3-73 filter.

Results

ANS Interaction with Poly-β-cyclodextrin. ANS in aqueous solution has an absorption maximum at 365 nm (27 400 cm^{-1}), an emission maximum at 556 nm (18 000 cm^{-1}), a lifetime of ~0.25 ns, and a quantum yield of ~0.004. Upon binding to PCD, the absorption spectrum is unchanged. However, the peak emission shifts to 496 nm (20 170 cm^{-1}), the lifetime is increased to 10 ns, and there is a 120-fold increase in the fluorescence yield. The binding of the ligand X (ANS) to the poly-β-cyclodextrin is represented by reaction 1 with the

$$\mathrm{PCD} + \mathrm{X} \underset{}{\overset{K_d}{\rightleftharpoons}} \mathrm{XPCD} \qquad (1)$$

dissociation constant shown in eq 2, where β is the degree of

$$K_d = \frac{[\mathrm{PCD}][\mathrm{X}]}{[\mathrm{XPCD}]} = \frac{1-\beta}{\beta}[\mathrm{PCD}] \qquad (2)$$

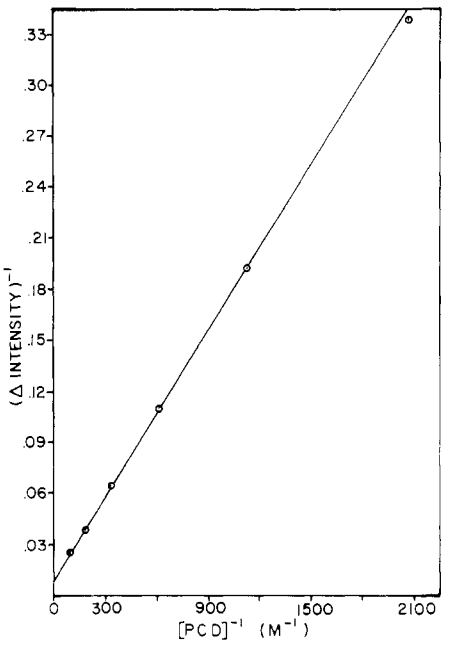

FIGURE 1: Titration of ANS by PCD, at 1 atm.

association of the ligand probe. In the case of ANS, the degree of association can be determined by eq 3, where ΔF stands

$$\beta = \frac{\Delta F}{\Delta F_\infty} = \frac{[\mathrm{XPCD}]}{[\mathrm{X}] + [\mathrm{XPCD}]} \qquad (3)$$

for the increase in fluorescence at a fixed PCD concentration and ΔF_∞ stands for the increase in fluorescence under conditions of complete binding of the fixed ANS concentration. The last two equations yield eq 4. [PCD] is the concentration

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_\infty}\left(1 + \frac{K_d}{[\mathrm{PCD}]}\right) \qquad (4)$$

of free sites of the polymer, but since the experiments are conducted under conditions of a large excess of binding sites over ANS, free PCD can be replaced by the total polymer, as monomer concentration, without sensible error. According to the last equation then, a plot of $1/\Delta F$ against $1/[\mathrm{monomer}]$ will yield an intercept $1/\Delta F_\infty$ and a slope $K_d/\Delta F_\infty$. The binding of a fixed concentration of ANS by increasing concentrations of polymer, at 1 atm, is shown in Figure 1. The fluorescence of ANS is enhanced by a factor of 120 upon binding, and the single slope indicates a virtually unique dissociation constant $K_d = 2 \times 10^{-2}$ M, or a free energy of association $\Delta G° = -2.3$ kcal. The observation of a single dissociation constant is in agreement with work of Harada et al. (1977), who employed 6-*p*-toluidinylnaphthalene-2-sulfonate.

Binding of ANS under Pressure. The fluorescence intensity from an equilibrium with $\beta = 0.13$ at atmospheric pressure decreases readily with increasing pressure, with most of the change occurring in the first few kilobars, as shown in Figure 2. This decrease could be due to unbinding of ANS or to some pressure-mediated quenching of bound ligand. If the latter were the case, the fluorescence lifetime should change in parallel with the intensity. As shown in the figure, the lifetimes measured by either phase or modulation are essentially constant in the pressure range of 0–8 kbar. The difference in the absolute values of about 3 ns indicates heterogeneity of the emitting species. Quite apart from the possibility of small differences among the complexes, reflected not in the

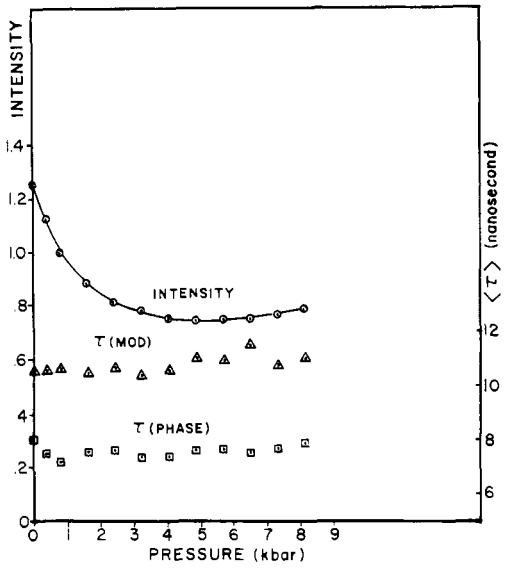

FIGURE 2: Pressure response of ANS + 3 × 10^{-3} M PCD: (○) intensity normalized at 1 kbar; (△) average fluorescence lifetime determined by modulation; (□) average fluorescence lifetime determined by phase.

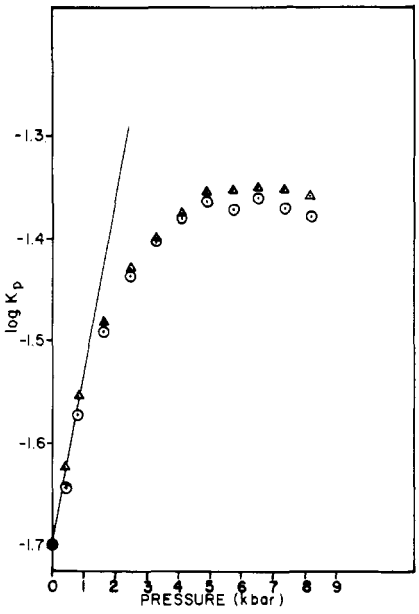

FIGURE 3: Dissociation constant vs. pressure for ANS + 3 × 10^{-3} M PCD: (○, △) identical experiments.

affinity for the polymer but only in the spectral properties, there exists in this case, as in all cases of strong dependence of the fluorescence spectrum upon the environment, a time-dependent relaxation that results in heterogeneity of the emitted lifetimes (Ware et al., 1968; Brand & Gohlke, 1973). At the degrees of association investigated, the contribution from free ANS is too small to lead to the appreciable heterogeneity observed.

To calculate the dissociation constant as a function of pressure, eq 4 is employed with [PCD] calculated from the starting concentration of polymer and the volume contraction under pressure. The standard volume change on formation of the complex at pressure p is given by eq 5. Notice that

$$\Delta V^\circ = RT\frac{\mathrm{d} \ln K_p}{\mathrm{d}p} \qquad (5)$$

the usual negative sign is missing in eq 5 because we are using

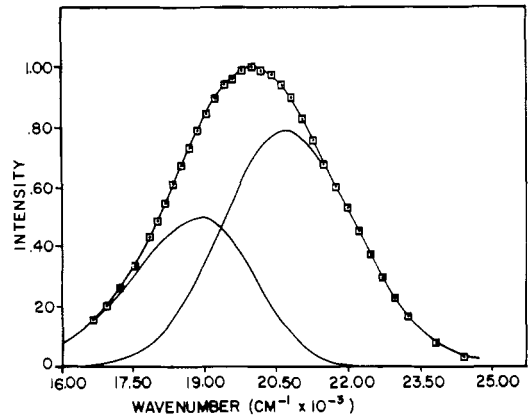

FIGURE 4: Emission spectrum of PRODAN + 3.6 × 10^{-4} M PCD at 1 atm: (□) corrected total intensity; (—) computer-separated spectra due to free and bound PRODAN.

the *dissociation* constant to calculate the standard volume change upon *association*. A plot of ln K_p vs. pressure is shown in Figure 3. K_p is seen to change rapidly with pressure in the first 3 or 4 kbar, while remaining virtually constant at the higher pressures. The initial slope gives a standard volume change of +9.3 mL upon formation of the complex.

PRODAN Interaction with Poly-β-cyclodextrin. PRODAN, like ANS, is a substituted naphthalene fluorophore, but its response to changes in the environment is markedly different. In water the maximum emission is at 531 nm (18 820 cm^{-1}). When PRODAN is bound to poly-β-cyclodextrin, the quantum yield increases by only a factor of 3, but the peak intensity shifts to 483 nm (20 750 cm^{-1}). This combination of not too dissimilar intensities and large peak shifts makes it possible to separate clearly the emission due to free and bound PRODAN and makes unnecessary the determination of fluorescence lifetimes to fix the degree of association. The intensity and spectral distribution are known for the free probe in water. The separation of the contributions of free and bound PRODAN was carried out with a computer program which gave the best fit to two skewed Gaussians, one of which had all parameters, except intensity, held constant. If this technique is valid, the peak position and half-width of the second curve should remain constant as its relative intensity changes during a titration of PRODAN with polymer at atmospheric pressure. This condition is observed to hold until the intensity difference gets so large that the entire fit fails, which happens when the area of one spectrum is about 10 times larger than that of the other. Figure 4 shows the separated curves and the experimental data for a polymer concentration of 3.6 × 10^{-4} M. The degree of association is taken to be proportional to the 483-nm peak, and the data over a range of polymer concentrations are analyzed according to eq 4. The results, shown in Figure 5, yield K_d = 8.4 × 10^{-5} M, or ΔG° = −5.6 kcal.

Binding of PRODAN under Pressure. The fluorescence maxima of both free and bound PRODAN shift to the red as pressure increases and reach 543 (18 410 cm^{-1}) and 500 nm (20 000 cm^{-1}), respectively, at the highest pressures. A typical result for 5.7 kbar is shown in Figure 6, and the complete pressure response is shown in Figure 7. As in the case of ANS, K_p changes rapidly with pressure at first and then reaches a constant value (Figure 8). The standard volume change upon formation of the complex is +9.2 mL.

Ligand Compressibility Curves. Conclusions. Let $v_L(p)$ and $v_w(p)$ stand for the volumes occupied at pressure p by the ligand and water, starting with 1 mL at atmospheric pressure. The change in volume that takes place when the ligand of

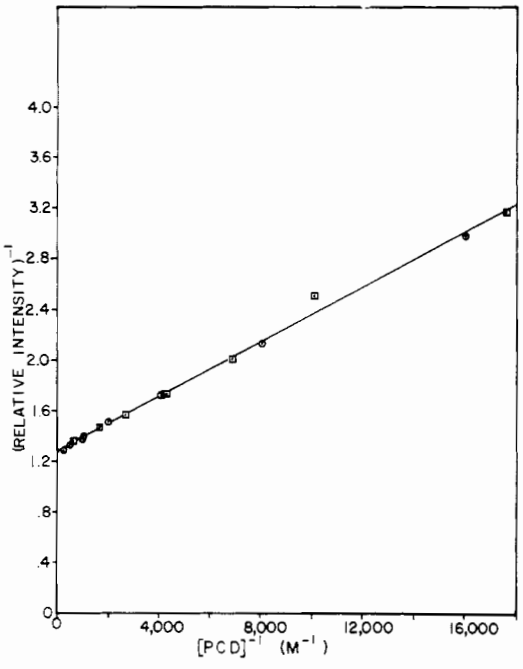

FIGURE 5: Titration of PRODAN by PCD at atmospheric pressure: (○, □) repeated experiments.

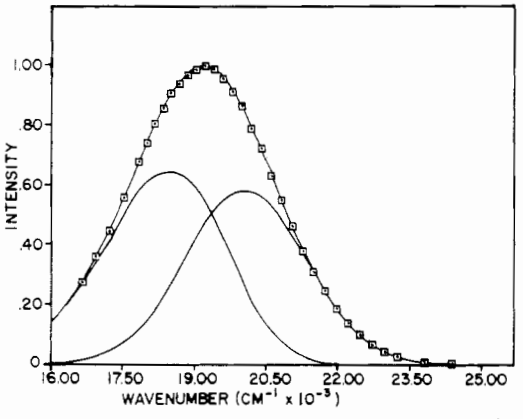

FIGURE 6: Emission spectrum of PRODAN + 3.6×10^{-4} M PCD at 5.7 kbar: symbols as in Figure 4.

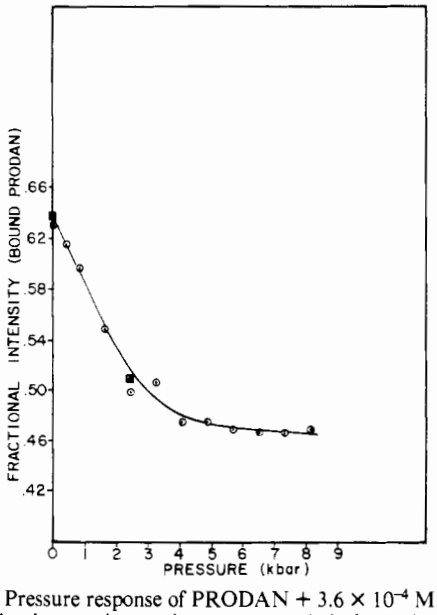

FIGURE 7: Pressure response of PRODAN + 3.6×10^{-4} M PCD: (○) data obtained upon increasing pressure; (□) data obtained upon releasing and reraising pressure.

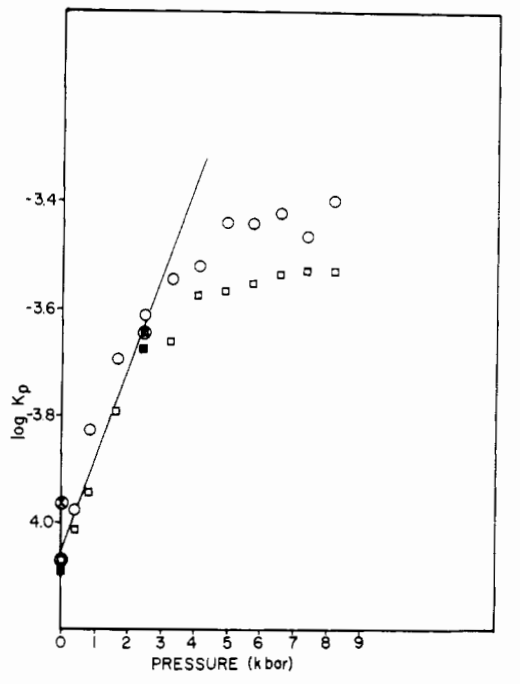

FIGURE 8: Dissociation constant vs. pressure for PRODAN + PCD: (□) 3.6×10^{-4} M PCD; (○) 5.4×10^{-4} M PCD; (■, ⊗) data obtained after releasing and reraising pressure.

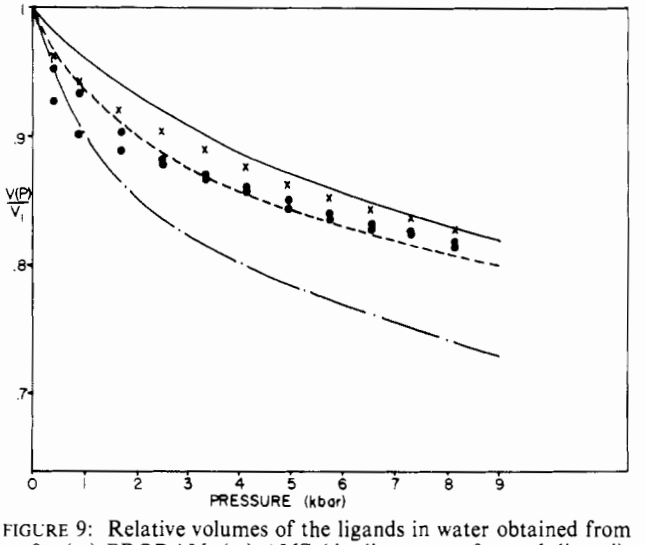

FIGURE 9: Relative volumes of the ligands in water obtained from eq 8: (●) PRODAN; (×) ANS (duplicate runs for each ligand). Curves are from the literature values for water (—), toluene (- - -), and hexane (·—·).

volume V_L at atmospheric pressure substitutes for water at an incompressible binding site is shown by eq 6. The standard

$$\Delta V_p = V_L[\nu_L(p) - \nu_w(p)] \quad (6)$$

free energies of formation of the complexes at pressures p and 1 atm, respectively ($\Delta G°_p$ and $\Delta G°_0$), are related through eq 7. Introducing the corresponding degrees of association at

$$\Delta G°_p - \Delta G°_0 = p\Delta V_p = pV_L[\nu_L(p) - \nu_w(p)] \quad (7)$$

pressures p and 1 atm, β_p and β_0, at constant concentration of binding sites the last equation becomes

$$\frac{RT}{pV_L} \ln \frac{(\beta_0^{-1} - 1)}{(\beta_p^{-1} - 1)} + \nu_w(p) = \nu_L(p) \quad (8)$$

In the last equation, V_L may be set equal to the molecular weight of the ligand without sensible error and $\nu_w(p)$ is accurately known from direct measurements (Bridgman, 1949). Consequently, this equation may be used to obtain $\nu_L(p)$, the

specific volume of the ligand molecularly dispersed in water. The physical realism of the proposed model can then be judged by comparison of $v_L(p)$ with the known values for organic liquids since the last equations would only hold if the compressibility of the binding site is neglected. Figure 9 shows a comparison of the compressibility curves for ANS and PRODAN obtained from the experimental degrees of association by means of eq 8, together with the directly measured curves for water, toluene, and hexane. The ANS curve is defined with better precision than that of PRODAN as the relative change in the degree of association with pressure is about 45% in the former compound and 27% in the latter. Comparison of the curves with the known bulk compressibilities of organic liquids and water reveals striking general similarities and characteristic differences. At low pressures the compressibilities of the ligands are what one would expect from their carbon–hydrogen skeleton: virtually an all-aromatic compressibility for ANS and a mixed aromatic and aliphatic one for PRODAN. The initial differential compressibilities are some 2 or 3 times larger than that of water but much smaller at higher pressures; they become equal or even a little smaller than water at 8 kbar. Believers in the "hydrophobic bond" could easily see these curves as being in broad agreement with the concept that water forms a tight cage around nonpolar compounds since the effect of the cage would be to increase the compressibilities at low pressure and decrease them at higher pressures. The charge of ANS and the appreciable dipole moment of PRODAN could also be invoked to explain these effects. In any case, it is evident that the compressibility curve of a substance molecularly dispersed in a solvent provides an important means of study of the molecular interactions and our experiments show that the equilibria of ligands with incompressible binding sites provide one method—not hitherto exploited—of establishing such compressibility curves.

The results have interest as regards the interpretation of the effects of high pressure upon proteins. The leveling off of the equilibrium at high pressure at values of the degree of association far from zero deserves special attention. In the studies of the effect of pressure upon a spectroscopic property of a protein, this leveling off has been uniformly interpreted as an indication that the corresponding chemical equilibrium was driven to completion and the results have been analyzed accordingly. Our observations, and the straightforward physical interpretation of the effect as arising from similarities in the differential compressibilities of water and ligand in the region of 6–8 kbar, give sufficient reason to doubt this assumption.

In conclusion, we have shown that for an incompressible binding site the experimental results of the ligand-site equilibria are in reasonable agreement with calculations based on the compressibilities of ligand and solvent. Although additional features are sure to complicate the effect of pressure upon the binding of ligands by proteins, a quantitative analysis along the lines outlined in this paper appears to be more promising and significant than the usual qualitative statements about the differential effects of pressure upon the several bond types.

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