Sparrow, J. P. (1976) *J. Org. Chem. 41*, 1350.
Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, p 53, W. H. Freeman, San Francisco, CA.
Tregear, G. W., van Rietschoten, J., Greene, E., Niall, H. D., Keutmann, H. T., Parsons, J. A., D'Riordan, J. H., & Potts, J. T., Jr. (1974) *Hoppe-Seyler's Z. Physiol. Chem. 355*, 415.
van Rietschoten, J., Granier, C., Rochat, H., Lissitzky, S., & Miranda, F. (1975) *Eur. J. Biochem. 56*, 35.
Westall, F. C., Scotchler, J., & Robinson, A. B. (1972) *J. Org. Chem. 37*, 3363.
Yamashiro, D., & Li, C. H. (1973) *J. Am. Chem. Soc. 95*, 1310.
Yamashiro, D., & Li, C. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4549.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1977) *Anal. Biochem. 77*, 569.

Light-Scattering Investigation of the Subunit Dissociation of *Homarus americanus* Hemocyanin. Effects of Salts and Ureas†

Theodore T. Herskovits,* Richard C. San George, and Lawson J. Erhunmwunsee

ABSTRACT: The subunit dissociation of the hemocyanin of the lobster, *Homarus americanus*, by the various salts of the Hofmeister series and the hydrophobic reagents of the urea–guanidinium chloride (GdmCl) class was investigated by laser light scattering molecular weight measurements. The dissociations of the hemocyanin dodecamers to hexamers by the various salts and the lower members of the urea series are found to be rapid and reversible, as predicted by the mass action law for monomer–dimer type of reactions. The salts are found to be very effective dissociating agents with the usual order of increasing effectiveness, $Cl^- < Br^- < I^- < ClO_4^-$, SCN^-. The ureas and GdmCl are found to be relatively ineffective dissociating reagents. In addition, the ureas show a decreasing order of effectiveness in going from urea to methyl-, ethyl-, and propylurea. This suggests that hydrophobic interactions are not the dominant stabilizing forces between the pairs of hexamers that form the dodecameric structure. Polar and ionic interactions appear to be the major stabilizing forces of the dodecameric structure. The use of equations derived for predicting the effects of dissociating reagents and salts on the structure of subunit proteins [Herskovits, T. T., & Ibanez, V. S. (1976) *Biochemistry 15*, 5715–5721] together with binding and Setschenow constants based on model amino acid data is found to give good account of the dissociation behavior observed with the salts, urea, and methylurea in the presence of calcium ion at both pH 7.8 and pH 9.5. The apparent number of amino acids at the contact areas of the hexamers, N_{app}, required to fit the dissociation data were found to be 24 ± 8 at pH 7.8 and 23 ± 4 at pH 9.5. However, because of the possible effects of molecular microheterogeneity, the estimates of amino acids at the contact areas must be viewed with caution, depending on further investigations.

The hemocyanins of the arthropod species are multisubunit proteins of varying complexity, assembled from one to eight basic hexameric units (Bijholt et al., 1979; Klarman et al., 1979; Jeffrey, 1979). Thus, the hemocyanins isolated from the hemolymph of the lobsters *Panulirus interruptus* and *Panulirus vulgaris* consist essentially of the single hexameric species (Kuiper et al., 1975; Van Bruggen et al., 1963) whereas the hemocyanin of the horseshoe crab, *Limulus polyphemus*, is an assemblage of six hexameric units (Bijholt et al., 1979). The hemocyanin of the lobster *Homarus americanus* has an intermediate structure, consisting of two basic hexameric units (Morimoto & Kegeles, 1971). With the hemocyanin of the ghost shrimp, *Callinassa californiensis* (Roxby et al., 1974), the lobster hemocyanin represents one of the few hemocyanins that exhibit ligand-mediated monomer-dimer type of association-dissociation equilibria.

Changes in solvent conditions, such as pH, the concentration of divalent ions, and the concentration of dissociating reagents, are known to alter the state of association of the different hemocyanin species. For the past several years, we have investigated the dissociation behavior of subunit proteins by using hydrophobic reagents and salts as probes of the contact areas of the subunits (Elbaum & Herskovits, 1974; Herskovits et al., 1977, 1980). In the case of the hemocyanin of the crab, *Callinectes sapidus*, light scattering molecular weight measurements have shown that the ureas and the Hofmeister series of salts dissociate both the hexameric and the dodecameric components of this hemocyanin (Herskovits et al., 1981). The salts were found to follow the usual order of effectiveness as dissociating agents of the dodecameric component whereas the ureas showed an inverse order of decreasing effectiveness with increasing hydrocarbon content of the urea, suggesting that polar and ionic interactions are relatively more important than hydrophobic interactions for the stabilization of the dodecameric structure. Unfortunately, the hemocyanin components of *Callinectes* were not found to exhibit true equilibrium behavior of the dissociation species, as suggested by the lack of concentration dependence of the light-scattering molecular weights upon dilution of the protein components. Such effects have been noted with most of the hemocyanins and have been attributed to the effects of microheterogeneity of the dissociating species (Di Giamberardino, 1967; Konings et al., 1969; Siezen & Van Driel, 1973). These findings did not allow us to analyze the dissociation data in any significant detail. The fact that the *Homarus* hemocyanin exhibits rapid and reversible dodecamer–hexamer equilibrium (Morimoto & Kegeles, 1971) and the desire to investigate the dissociation behavior of other hemocyanins regarding the question of the forces that hold the subunits together have prompted the

† From the Department of Chemistry, Fordham University, Bronx, New York 10458. *Received August 19, 1980.* This work was supported by Grant AM 20156 from the National Institutes of Health, U.S. Public Health Service.

0006-2960/81/0420-2580$01.25/0

present light-scattering study.

Materials and Methods

Homarus hemocyanin was isolated from live Maine lobsters purchased at the local fish market. The serum was collected from the severed hind legs of several lobsters in beakers placed on ice. The serum was allowed to clot overnight at 4 °C,.after which the clotting material was removed by centrifugation at 15000 rpm for approximately 10 min in the cold. The pooled crude hemocyanin was filtered through Whatman filter paper and dialyzed against 0.1 M NaCl and 0.05 M Tris,[1] pH 7.8, containing 0.01 M $CaCl_2$. Further purification was effected by chromatography on Bio-Gel A-5m (200–400 mesh) columns equilibrated with the same solvent system at 4 °C. Usually, 10–15 mL of the crude hemocyanin was processed on 35 × 4.0 cm columns. The hemocyanin was collected over a period of 2 days at a flow rate of 8–12 mL/h. The elution profiles showed a major hemocyanin component with some minor leading and trailing material. Approximately 70–80% of the hemocyanin eluting as the major peak was pooled and used usually within 1–2 weeks. Hemocyanin free of calcium was prepared by dialysis in the cold against 0.01 M EDTA and 0.05 M Tris buffer, pH 7.8, followed by 0.1 M NaCl and 0.05 M Tris, pH 7.8. For the alkaline pH experiments, glycine–sodium hydroxide buffer of 0.1 ionic strength (pH 9.5) was used as solvent and dialysate.

Hemocyanin concentrations were based on absorbance measurements with a Cary 14 recording spectrophotometer, using the "percent" extinction coefficient $E^{1\%}_{278\ nm} = 13.4$ at pH 7.8 in the presence of 0.01 M Ca^{2+}. The same extinction value was used for pH 9.5–9.6 measurements, reported by Morimoto & Kegeles (1971). For concentration determinations in the presence of some of the ureas and salts, the slight turbidity of the solutions required measurements to be made in the presence of 6.0 M GdmCl. For these measurements, an $E^{1\%}_{278\ nm}$ value of 12.6 was obtained in the presence of 6.0 M GdmCl. Our extinction coefficients were based on dry weight determinations on hemocyanin solutions dialyzed exhaustively against 0.03 M NaCl and dried at 100–105 °C. The contribution of NaCl to the dry weight was similarly determined, using the dialysate, and subtracted from the weights of the protein solutions. Absorbance measurements were made on a portion of the dialyzed hemocyanin solution after appropriate dilution with the calcium ion containing solvents of this study.

All the reagents and salts employed were analytical or reagent grade materials. The urea was ultrapure grade purchased from Schwarz/Mann and used without further purification. The alkylureas were purified further by recrystallization from hot ethanol.

Light scattering measurements were made with a Chromatix KMX-6 laser light-scattering instrument at 632.8 nm, using the angular setting of the annulus at 6–7° and a field stop of 0.2-mm diameter. The light scattering data were analyzed by using

$$(M_w)_{app}^{-1} = Kc/\bar{R}_\theta = 1/M_w + 2B'c \qquad (1)$$

where $\bar{R}_\theta$ is the excess Rayleigh factor representing the light scattering of a given solution minus the scattering of the solvent, M_w is the weight-average molecular weight, B' is the second virial coefficient, c is the hemocyanin concentration, and K is the optical constant (Kaye & Havlik, 1973) given by

$$K = 2\pi^2 n^2 (\partial n/\partial c)_\mu^2 (1 + \cos^2\theta)/(\lambda^4 N)$$

In the latter constant, n is the refractive index of the solvent, $(\partial n/\partial c)_\mu$ is the refractive index increment at constant chemical potential of the diffusible component obtained on dialyzed solutions, λ is the wavelength of light, and N is Avogadro's number. The light-scattering data were corrected for depolarization based on the depolarization ratio, $\sigma = 0.0051$, measured at 90° in a Wood light-scattering photometer. The Cabannes correction $C(\theta) = 1.006$ was applied to all our data. This value was calculated by using the equation appropriate for the instrument, given by Kaye & Havlik (1973). Solvent and protein solutions were filtered through a set of two 13-mm diameter Swinnex filters in series, utilizing 0.2-μm Gelman metricel membrane filters. Light-scattering measurements were usually made on solutions diluted serially in 5-mL volumetric flasks by using appropriate volumes of hemocyanin stock solution, buffered solvent, and concentrated reagent (2–5 M).

The refractive index increments, $(\partial n/\partial c)_\mu$, and the refractive indexes of the various solvents used were measured in a Wood light scattering photometer equipped with a differential refractometer at 630 nm, as previously described (Elbaum & Herskovits, 1974). Since the light-scattering measurements were made at a slightly higher wavelength of 632.8 nm, a small dispersion correction should be applied to both our n and $(\partial n/\partial c)_\mu$ data listed in Table I. In the region of 546 to 633 nm, $(\partial n/\partial c)_\mu$ data show a very small dispersion of about 0.002 g^{-1} cm^3 (Chincholi, et al., 1974; Eisenberg et al., 1977; Jolly & Eisenberg, 1976), comparable to the experimental uncertainty of our data, 0.003 g^{-1} cm^3. The actual error calculated by using the disperion equation of Perlmann & Longsworth (1948), $(\partial n/\partial c)_\lambda = (\partial n/\partial c)_{578}\,[0.940 + 2.00 \times 10^4 nm^2/\lambda^2]$ with a $(\partial n/\partial c)_{578} = 0.182$ g^{-1} cm^3, gives estimates that are 8×10^{-5} g^{-1} cm^3 lower at 632.8 nm than at 630 nm, which can be clearly ignored. The refractive indexes of the solvents, n at 632.8 nm, were estimated by using the measured refractive index differences, Δn, obtained with water as a reference, and the refractive index of water, $n_w = 1.3317$, obtained by interpolation of wavelength dependence data of Kruis (1936), with $n = 1.3317 + \Delta n$. Any intermediate or higher values of the $(\partial n/\partial c)_\mu$ and n estimates given in Table I were obtained by interpolation of the values listed. The hemocyanin solutions of about 0.5–1.0% used for these experiments were prepared by dialysis against the appropriate solvents for 1–3 days in the cold, as previously described (Elbaum & Herskovits, 1974).

Results

Concentration Dependence of Light-Scattering Data. The light-scattering data of *Homarus* hemocyanin measured as a function of concentration are shown in Figures 1 and 2. The results obtained give typical $Kc/\bar{R}_\theta$ vs. c plots, characteristic of subunit protein systems that exhibit dissociation at low protein concentration dictated by the stoichiometry of the protein reaction. Such behavior of the system produces the upswing in the data plots at low protein concentrations (Elbaum & Herskovits, 1974; Herskovits et al., 1978). We have been able to fit all our data obtained at both pH 7.8 and pH 9.5 and in the presence of dissociating agents such as urea, NaBr, and NaI by using the light scattering equation appropriate for dimer–monomer type of dissociation

$$(M_w)_{app}^{-1} = Kc/\bar{R}_\theta = 1/[M_{12}(1 - 1/2\alpha)] + 2B'c \qquad (2)$$

where M_{12} is the molecular weight of the dodecameric form of the protein, and α is the weight fraction of the protein that dissociates to hexamers. In fitting the data, the fraction of

[1] Abbreviations used: GdmCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane; μ, ionic strength; EDTA, ethylenediaminetetraacetic acid.

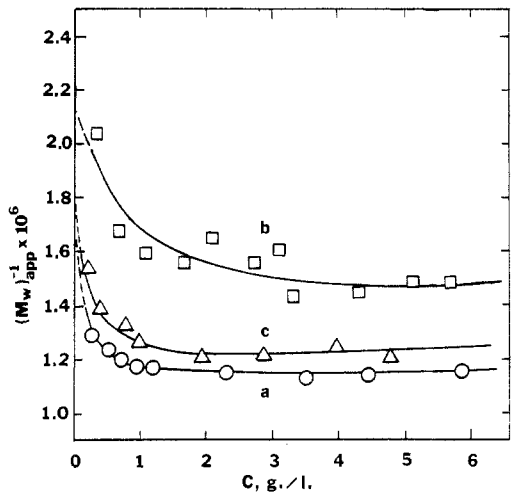

FIGURE 1: Concentration dependence of the light scattering of *Homarus americanus* hemocyanin at pH 7.8 (0.1 M NaCl and 0.05 M Tris) plotted according to eq 2 and 3. (a) No added reagent; computed with $K_D = 2 \times 10^{-7}$ M^{-1} and $B' = 5 \times 10^{-9}$ L mol g^{-2}. (b) 2 M urea; computed with $K_D = 8.5 \times 10^{-6}$ M^{-1} and $B' = 1 \times 10^{-8}$ L mol g^{-2}. (c) 2 M NaBr plus 0.01 M Ca^{2+}; computed with $K_D = 7 \times 10^{-7}$ M^{-1} and $B' = 1 \times 10^{-8}$ L mol g^{-2}.

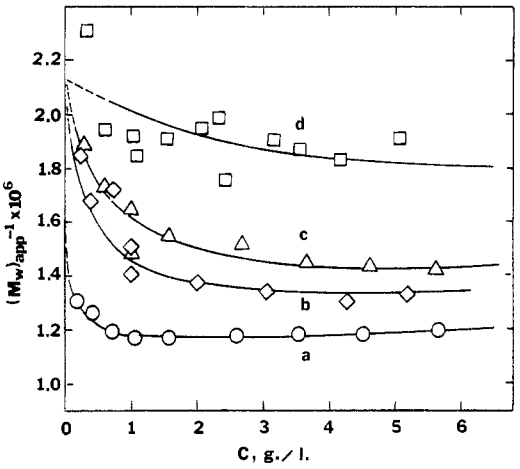

FIGURE 2: Concentration dependence of the light scattering of *Homarus americanus* hemocyanin at pH 9.5 (μ = 0.1 glycine and 0.02 M Ca^{2+}) plotted according to eq 2 and 3. (a) No added reagent; computed with $K_D = 1.8 \times 10^{-7}$ M^{-1} and $B' = 7.5 \times 10^{-9}$ L mol g^{-2}. (b) 3 M NaCl; computed with $K_D = 2.5 \times 10^{-6}$ M^{-1} and $B' = 1 \times 10^{-8}$ L mol g^{-2}. (c) 2 M NaBr; computed with $K_D = 7.4 \times 10^{-6}$ M^{-1} and $B' = 6.7 \times 10^{-9}$ L mol g^{-2}. (d) 3M urea; computed with $K_D = 5 \times 10^{-5}$ M^{-1} and $B' = 1 \times 10^{-8}$ L mol g^{-2}.

protein that dissociates is calculated by use of the equilibrium constant, defining α at any given protein concentration c, where

$$K_D = \frac{4c\alpha^2}{(1-\alpha)M_{12}} \qquad (3)$$

The data of Figures 1 and 2 were fitted with $K_D = 1.8 \times 10^{-7}$ to 5×10^{-5} M^{-1} and $B' = 5 \times 10^{-9}$ to 1×10^{-8} L mol g^{-2}. The method outlined by Schroder et al. (1976) was used in some cases to aid the calculations and fitting of the data. The satisfactory fit of the concentration dependence data suggests that the major species present at the solvent conditions where there is sufficient dissociation are the parent dodecamers in equilibrium with "half-mers" or hexamers. The presence of two species sedimenting in the ultracentrifuge with 17 and 25 S at alkaline pH of 9.4 to 9.7 was clearly established by Morimoto & Kegeles (1971), who have also shown that these two species are in dynamic equilibrium.

Effects of Ureas and Salts on the Dissociation. The molecular weight data obtained with various ureas and salts as a function of reagent concentration are shown in Figures 3–6. The higher alkylureas such as ethyl- and propylurea are found to be relatively ineffective dissociating agents when compared to the effects of some of the Hofmeister salts and to urea itself. The data of these figures studied at both pH 7.8 and pH 9.5 were obtained at a fixed protein concentration of approximately 1 g/L. The effects of nonideality are relatively small at this concentration (1–2%). We have actually corrected the apparent molecular weights by using eq 1 with a B' value of 1×10^{-8} L mol g^{-2}.

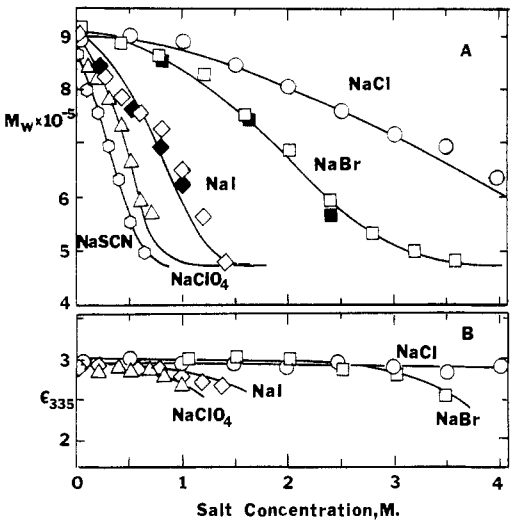

FIGURE 3: Effects of various salts on the molecular weight (M_w) and the absorbance at the 335-nm copper band (ϵ_{335}) of *Homarus* hemocyanin at pH 9.5 and 0.02 M Ca^{2+}. The molecular weight data as a function of salt concentration were fitted by using eq 6 and 7 and some of the parameters listed in Table II. Data represented by filled symbols were obtained on hemocyanin initially exposed to 3.2 M NaBr and 1.0 M NaI. The best fit curves shown were computed with the following parameters: NaBr, $K_w = 2 \times 10^{-8}$ M^{-1} $N_{app} = 24$; NaI, $K_w = 3 \times 10^{-8}$ M^{-1}, $N_{app} = 16$; $NaClO_4$, $K_w = 5 \times 10^{-8}$ M^{-1}, $N_{app} = 24$; NaSCN, $K_w = 2 \times 10^{-7}$ M^{-1}, $N_{app} = 28$; NaCl, $K_w = 4 \times 10^{-8}$ M^{-1}, $2N_{app}K_B = 1.32$. Protein concentration: 1 g/L; solvent: μ = 0.1, pH 9.5, glycine buffer and 0.02 M $CaCl_2$.

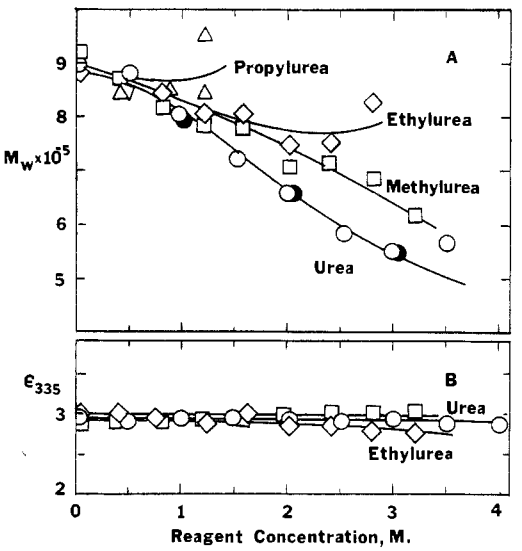

FIGURE 4: Effects of various ureas on the molecular weight (M_w) and the absorbance at the 335-nm copper band (ϵ_{335}) of *Homarus* hemocyanin at pH 9.5, 0.02 M Ca^{2+}, plotted as a function of reagent concentration. Data represented by filled circles were obtained on hemocyanin initially exposed to 4 M urea. The urea and methylurea curves were computed by using eq 6 and 7, with $K_w = 1 \times 10^{-7}$ M^{-1} and $N_{app} = 28$ for urea and $K_w = 7 \times 10^{-8}$ M^{-1} and $N_{app} = 17$ for methylurea. Protein concentrations and solvent are the same as in Figure 3.

Table I: Refractive Index and Refractive Index Increment Data of *Homarus americanus* Hemocyanin Obtained with Various Ureas and Salts at 25 ± 1 °C[a]

solvent[b]	$n_{632.8}$[a] (pH 9.5)[b]	$(\partial n/\partial c)_\mu$ pH 7.8[b]	$(\partial n/\partial c)_\mu$ pH 9.5[b]	solvent	$n_{632.8}$[a] (pH 9.5)[b]	$(\partial n/\partial c)_\mu$ (pH 9.5)[b]
aqueous pH 7.8[b]	1.335	0.180 ± 0.003	0.180 ± 0.003			
aqueous pH 9.5[b]	1.335					
1.0 M urea	1.340	0.176	0.175	1.0 M NaCl	1.342	0.172
2.0 M urea	1.346	0.170	0.170	2.0 M NaCl	1.348	0.170
3.0 M urea	1.351		0.170	3.0 M NaCl	1.354	0.163
4.0 M urea	1.364		0.157	4.0 M NaCl	1.360	0.157
1.0 M methylurea	1.340		0.179	1.0 M NaBr	1.344	0.171
2.0 M methylurea	1.349		0.169	2.0 M NaBr	1.353	0.162
3.0 M methylurea	1.357		0.163	3.0 M NaBr	1.362	0.154
1.0 M ethylurea	1.343		0.175	4.0 M NaBr	1.373	*c*
2.0 M ethylurea	1.352		0.169	0.4 M NaI	1.340	0.179
1.0 M propylurea	1.345		0.172	0.8 M NaI	1.345	0.173
2.0 M propylurea	1.355		*c*	1.2 M NaI	1.351	0.172
0.4 M GdmCl	1.340		0.176	1.6 M NaI	1.357	
0.8 M GdmCl	1.345		0.174	0.5 M $NaClO_4$	1.338	0.185
1.2 M GdmCl	1.350		0.164	0.8 M $NaClO_4$	1.339	0.185
0.4 M NaSCN	1.340		0.187	1.5 M $NaClO_4$	1.343	*c*
0.8 M NaSCN	1.344		0.191			
1.2 M NaSCN	1.349					

[a] Calculated by use of the formula $n_{632.8} = 1.3317 + \Delta n$ (see Materials and Methods for detail). [b] Buffered solvents, 0.1 M NaCl, 0.05 M Tris, pH 7.8, 0.01 M $CaCl_2$, and 0.1 ionic strength, pH 9.5, glycine buffer containing 0.02 M $CaCl_2$. [c] Protein solutions formed precipitates at the concentrations of 0.5–1.0% employed.

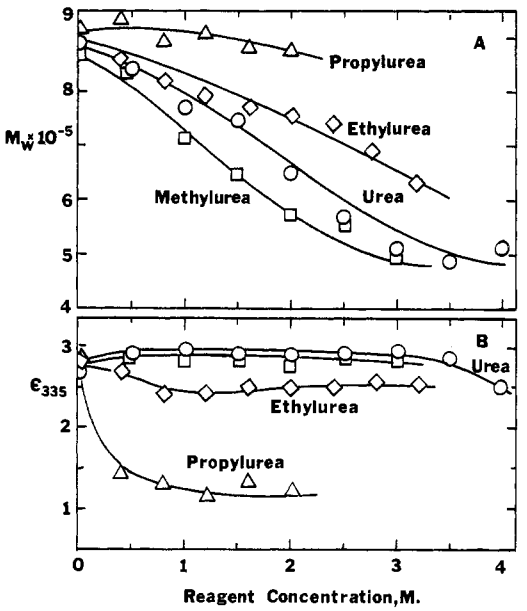

FIGURE 5: Effects of various ureas on the molecular weight and the absorbance at the 335-nm copper band (ϵ_{335}) of *Homarus* hemocyanin at pH 7.8 plotted as a function of reagent concentration. The urea and the methylurea curves were computed by using eq 6 and 7, with $K_w = 1 \times 10^{-7}$ M^{-1} and $N_{app} = 28$ for urea and $K_w = 2 \times 10^{-7}$ M^{-1} and $N_{app} = 25$ for methylurea. Protein concentration: 1 g/L; solvent: 0.1 M Cl^- and 0.05 M Tris buffer, pH 7.8.

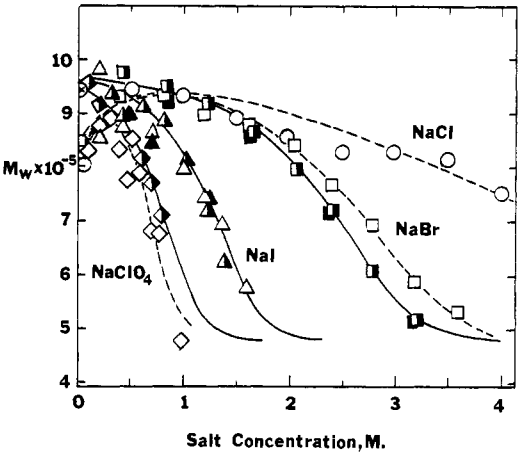

FIGURE 6: Effects of various salts on the molecular weight (M_w) of *Homarus* hemocyanin at pH 7.8 (0.05 M Tris) plotted as a function of salt concentration. Data represented by open symbols were obtained on solutions containing no calcium, while that data points represented by half-filled symbols were obtained on solutions containing 0.01 M Ca^{2+}. Filled symbols represent data obtained on solutions initially exposed to 3.2 M NaBr and 1.4 M NaI. The best-fit curves represented by the solid lines were computed for the 0.01 M Ca^{2+} data (see Discussion) by using eq 6 and 7 with the following parameters: NaBr, $K_w = 3 \times 10^{-10}$ M^{-1}, $N_{app} = 35$; NaI, $K_w = 1 \times 10^{-9}$ M^{-1}, $N_{app} = 17$; $NaClO_4$, $K_w = 6 \times 10^{-9}$ M^{-1}, $N_{app} = 21$; $c = 1$ g/L.

The dissociation data of subunit proteins can be analyzed by using eq 4, derived to account for the effects of dissociating reagents on the protein equilibria (Herskovits & Ibanez, 1976)

$$K_D = K_w\{1 + K_B[D]\}^{mN_{app}} \simeq K_w \exp(mN_{app}K_B[D]) \quad (4)$$

where K_D and K_w represent the dissociation constants in the presence and the absence of dissociating reagent, [D] is the concentration of the dissociating reagent, m is the number of subunits or fragments formed upon dissociation, N_{app} is the apparent number of amino acids at the subunit contacts that become exposed as a result of dissociation, and K_B is the binding or interaction constant of the average amino acid with the dissociating reagent. The N_{app} and K_w parameters of eq 4 can be extracted from the molecular data by using the logarithmic form of eq 4 on the RT scale (with $m = 2$)

$$\Delta G°_D = \Delta G°_w - 2RTN_{app} \ln (1 + K_B[D])$$

$$\simeq \Delta G°_w - 2N_{app}RTK_B[D]$$

$$= -RT \ln K_D \quad (5)$$

Alternatively, it is possible to fit the molecular weight data by using eq 6 and the combined expressions 3 and 4, given as eq 7 (Herskovits et al., 1978), where

$$M_w = M_{12}(1 - 1/2\alpha) \quad (6)$$

and

$$\alpha^2/(1 - \alpha) = [K_wM_{12}/4c] \exp(2N_{app}K_B[D]) \quad (7)$$

Table II: Summary of the Dissociation Data of *Homarus americanus* Hemocyanin Dodecamers to Hexamers Based on Equations 5 and 9

reagent	K_B[a]	K_s[a]	slope (s) (kcal mol^{-1} M^{-1})	ΔG_w° (kcal mol^{-1})	$\Delta\bar{\nu}_{x,pref}$[b]	N_{app}
			0.16 M glycine, pH 9.5, 0.02 M $CaCl_2$[c]			
urea	0.032		−1.07	9.60	2.6	28
methylurea	0.042		−0.84	9.65	1.8	17
ethylurea	0.061		−0.78	9.70		
propylurea	0.108		~ −0.30	~9.0		
NaCl	0.014	−0.006	−0.81	10.0	1.2	
NaBr	0.053	−0.023	−1.52	10.6	1.1	24
NaI	0.18	−0.078	−3.32	10.2	0.5	16
$NaClO_4$	0.175	−0.076	−5.00	10.0	0.8	24
NaSCN	0.16	−0.070	−5.26	9.2	0.8	28
			0.05 M Tris, pH 7.8, 0.01 M $CaCl_2$[c]			
urea	0.032		−1.18	11.9	2.6	31
methylurea	0.042		−0.87	11.7	2.9	18
NaBr	0.053	−0.023	−2.20	12.9	2.0	35
NaI	0.18	−0.078	−3.62	12.1	1.7	17
$NaClO_4$	0.175	−0.076	−4.4	11.2	1.3	21
GdmCl	0.20	−0.088	−1.4	12.1	0.9	
			0.05 M Tris, pH 7.8, 0.1 M NaCl			
urea	0.032		−1.07	9.52		28
methylurea	0.042		−1.24	9.32		25
ethylurea	0.061		−0.89	9.95		
propylurea	0.108		−1.12	11.7		

[a] K_B and K_s values are from Herskovits et al. (1977, 1978). The K_B values of most of the salts are based on the Setschenow constants, K_s, of a peptide plus a methylene group value where $K_B = -2.303\ \Sigma K_s$. [b] Based on the initial linear portion of the ln K_D vs. ln a_x plots. [c] The urea and methylurea solutions also contained 0.1 M NaCl.

Our studies of hemoglobin dissociation by the Hofmeister salt series have shown that the required K_B constants to be employed with eq 4–6 can be approximated by using the relationship[2]

$$K_B = -2.303K_s \tag{8}$$

where K_s represents the Setschenow constant of a peptide unit and a methylene group, used to represent the interaction between the average amino acid at the protein–protein contact of the subunits and the particular salt involved (Herskovits et al., 1977, 1978).[3]

In deriving eq 4 and 5, weak binding with equal and independent binding sites was assumed (Herskovits & Ibanez, 1976). It is also possible to analyze dissociation data without any assumption regarding the nature of the interactions. The expression obtained by Tanford (1969) for multicomponent systems based on Wyman's theory of linked functions can be used.

$$\frac{d \ln K_D}{d \ln a_x} = \Delta\bar{\nu}_{x,pref} \tag{9}$$

In eq 9

$$\Delta\bar{\nu}_{x,pref} = \Delta\bar{\nu}_x - m_x/m_w\Delta\bar{\nu}_w$$

and a_x is the activity of the dissociating reagent or salt, $\Delta\bar{\nu}_{x,pref}$ represents the difference in preferential binding of solvent components at the two end states of the protein reaction, $\Delta\bar{\nu}_x$ and $\Delta\bar{\nu}_w$ are the differences between the number of molecules of reagent or salt and water molecules bound at the two protein states, and m_x and m_w are the molalities of the reagent or salt and water, respectively.

The data of Figure 7A,B show some of our results plotted according to eq 5 and 9, with the derived intercept, slope, $\Delta\bar{\nu}_{x,pref}$ and N_{app} parameters listed in Table II. The $\Delta\bar{\nu}_{x,pref}$

[2] This relationship between K_B and K_s was derived by assuming that the solubilizing effects of salts are due to soluble complex formation between the salt and the average peptide group (Herskovits et al., 1977). The equilibrium constant for the peptide–salt reaction in terms of the solubilities, S and S_0, of the average peptide group in the presence and in the absence of salt is given by the expression $K_B = [(S/S_0) - 1]/[D]$ (Herskovits et al., 1970). Combined with the logarithmic form of eq 5, we obtain

$$\Delta G^\circ{}_D = \Delta G^\circ{}_w - 2RTN_{app} \ln (S/S_0)$$

and with the logarithmic expression defining the Setschenow constant, K_s

$$\log (S/S_0) = -K_s[D]$$

we can write the free energy expression in the form

$$\Delta G^\circ{}_D = \Delta G^\circ{}_w + 2.303(2RTN_{app}K_s[D])$$

Comparing this last result with eq 5 of the text shows that

$$K_B = -2.303K_s$$

The satisfactory agreement of the N_{app} estimates of human hemoglobin A, obtained with the ureas and salts as probes by using this relationship (Herskovits et al., 1977, 1978), supports this conclusion.

[3] The problem of specific binding as opposed to nonspecific solvent effects regarding the interactions between biopolymers and salts have been discussed by various authors [see, for example, Robinson & Jencks (1965), Schrier & Schrier (1967), St. Pierre & Jencks (1969), von Hippel & Schleich (1969), and Herskovits et al. (1978)] with various emphasis regarding the detailed mechanism of interaction. As we have concluded in the previous paper of this series (Herskovits et al., 1978), we have no conceptual problem with the salts that solubilize or "salt in" the average peptide unit of the protein. Problems are encountered, however, with salts such as KF or Na_2SO_4 that interact unfavorably or "salt out" model compounds. In the latter cases, we must clearly resort to "nonspecific solvent effects" (Robinson & Jencks, 1965; St. Pierre & Jencks, 1969) as opposed to a "binding" mechanism of salt–biopolymer interaction for the explanation of the observed phenomena of salt-mediated protein reactions.

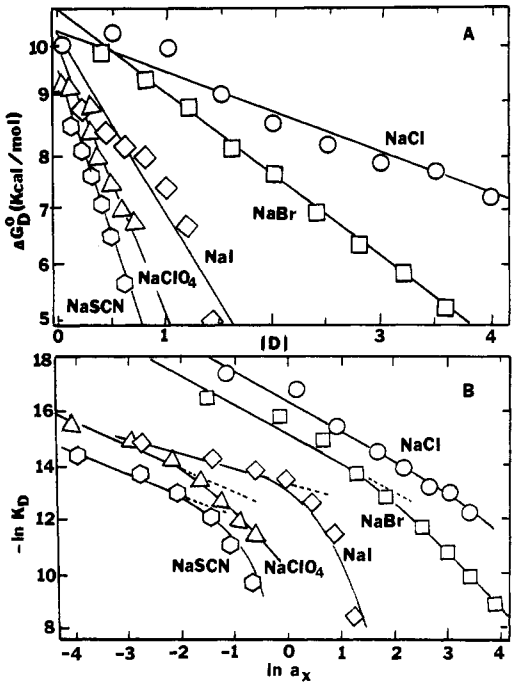

FIGURE 7: Plots of $\Delta G°_D$ vs. [D] and ln K_D vs. ln a_x for *Homarus* hemocyanin dodecamer to hexamer dissociation at pH 9.5 (0.02 M Ca^{2+}) based on eq 5 and 9. The derived intercepts, $\Delta G°_w$, slopes, $\Delta\bar{\nu}_{x,pref}$, and s and the related N_{app} parameters are listed in Table II.

estimates of about 0.5–3 mol of salt or urea bound per mol of hemocyanin were based on the slopes of the initial linear portions of the ln K_D vs. ln a_x plots, represented by the solid and continuing dashed lines in Figure 7B. The activity coefficients used for our calculations were taken from the data compiled by Robinson & Stokes (1959), Scatchard et al. (1938), and Aune & Tanford (1969). In the absence of activity coefficients for methylurea, the urea values were used for the latter calculations of the activities.

Details regarding the treatment of molecular weight data by using eq 5–7 are described in previous publications from our laboratory (Herskovits & Ibanez, 1976; Herskovits et al., 1977, 1978). The fraction of protein α that dissociates under any given condition required to estimate K_D (eq 3) was calculated by using the relationship

$$\alpha = 2(1 - M_w/M_{12}) \quad (10)$$

The accepted molecular weight for arthropod hemocyanin, $M_{12} = 9.4 \times 10^5$ (Ellerton et al., 1970; Morimoto & Kegeles, 1971), was used for our calculations involving the pH 9.5 data in the presence of 0.02 M Ca^{2+} and the pH 7.8 data in the absence of Ca^{2+} ions. The fit of our pH 7.8 data in the presence of 0.01 M Ca^{2+} required a slightly higher molecular weight of about 2%, with $M_{12} = 9.6 \times 10^5$.

Reversibility and Denaturation. The dissociations of *Homarus* hemocyanin dodecamers to hexamers were found to be relatively rapid, with essentially no observable changes in turbidity after about 10–15 min following mixing and filtration of the solutions. Turbidity changes were seen with some of the solutions at moderate to high reagent concentrations such as 1.6–2.0 M NaI and above 2 M propylurea and 3 M ethylurea. At these reagent concentrations, we see some evidence of denaturation, suggested by the changes in absorbance at the 335-nm copper band (see Figures 3 and 5B). The light-scattering data obtained in these regions of reagent concentration were not included in our analysis of the data based on eq 5–7.

The reversibility of the dodecamer to hexamer dissociation reaction was tested with several of the reagents. The data obtained are represented by the filled symbols in Figures 3, 4, and 6. The dissociation is clearly reversible since the molecular weights and $\Delta G°_D$ values fall on the expected curves predicted by eq 5–7. The reversibility data were obtained on solutions exposed to the highest reagent concentrations indicated in the figures (i.e., 4 M urea, 3.2 M NaBr, etc.) by using 0.4% hemocyanin and diluted to 0.1% with the appropriate amount of buffer and concentrated reagent. For some experiments, the reagent was removed by dialysis in the cold against reagent free buffer.

Discussion

The molecular weight data of this study obtained with the hemocyanin of the lobster *Homarus americanus* indicate that the dodecameric protein of this species can be dissociated to half-molecules of hexamers by the salts of the Hofmeister series and to a lesser extent also by some of the hydrophobic reagents of the urea–GdmCl class without any significant denaturation or unfolding of the subunits. The effectiveness of the salts as dissociating reagents shown in Figures 3 and 6 closely parallels their effects on the solubility of model amino acids (Schrier & Schrier, 1967; Nandi & Robinson, 1972a,b; von Hippel & Schleich, 1969).

A measure of effectiveness of the salts as dissociating agents is the Setschenow constants and the related binding constants, K_B, derived from the solubility data (Herskovits et al., 1977). The latter constants show close correlation with slope parameter, s, derived from $\Delta G°_D$ vs. [D] plots, based on eq 5 (Table II). Unlike the Hofmeister salts that seem to follow the expected order of effectiveness, with Cl^- at one end of the spectrum and I^- and SCN^- ions at the other end, the ureas shown a decreasing order of effectiveness with increasing hydrocarbon content in going from urea to ethyl- and propylurea. This suggests that hydrophobic effects are perhaps less important to the overall stability of the dodecameric structure of the hemocyanin than polar and electrostatic interactions. The importance of the latter forces for the stabilization of subunit proteins and virus assemblies is becoming increasingly apparent from the more recent literature [reviewed by Perutz (1978)].

The concentration dependence of the light-scattering data obtained with several of the dissociating agents shown in Figures 1 and 2 indicates that at moderate ranges of reagent concentration where there is no denaturation the dissociation behavior of *Homarus* hemocyanin is adequately described by the dodecamer–hexamer reaction scheme, dictated by the apparent equilibrium expression 3. Below the protein concentrations of 1 g/L, the light-scattering data obtained on this hemocyanin with no dissociating reagent present (data points represented by open circles in Figures 1 and 2) also exhibit dissociation required by the mass action law, with the ensuing upturn in the $(M_w)_{app}^{-1}$ vs. c plots. Together with the reversibility data of Figures 3 and 6, these results are consistent with the findings of Morimoto & Kegeles (1971) showing that the dodecameric and hexameric species of *Homarus* hemocyanin are in dynamic equilibrium. The dissociation behavior of this hemocyanin is very different from that observed with other arthropod hemocyanins such as the *Callinectes sapidus* protein similarly investigated by means of light scattering (Herskovits et al., 1981). The latter hemocyanins exhibit no changes in molecular weights or the relative concentrations of species present as a result of dilution of the proteins. This lack of apparent equilibrium among the hemocyanin species has been attributed to microheterogeneity of closely similar molecular species undergoing association–dissociation at slightly different concentrations or pH (Di Giamberardino,

1967; Konings et al., 1969; Siezen & Van Driel, 1973; Van Holde et al., 1977). Moderate effects of microheterogeneity of the *Homarus* hemocyanin should not be ruled out, in which case the observed equilibrium constant may actually represent some average value of this constant, characterizing the dissociation behavior of a closely related group of molecular species (Van Holde et al., 1977).

The effectiveness of the Hofmeister salts as dissociating agents has encouraged a more detailed analysis of some of the molecular weight data based on eq 4. As is shown in Figure 3, eq 6 and 7 give a fairly satisfactory account of the molecular weight data obtained at pH 9.5 in the presence of 0.02 M $CaCl_2$. The binding constants, K_B, used for the fit of our data are based on the solubility data of model compounds obtained by Nandi & Robinson (1972a,b), described in a previous study on human hemoglobin dissociation (Herskovits et al., 1977). We have assumed that the interaction of the average amino acid with a salt can be represented as the sum of a peptide and a single methylene group contribution to K_B, based on the Setschenow constants, K_s, of such groups.[2] Despite the number of uncertainties that the use of these constants entail, in the case of human hemoglobin, the estimates of the number of amino acids at the $\alpha_1\beta_2$-contact areas of the subunits obtained with the more effective salts were found to be in fairly satisfactory agreement with the estimates of 19 groups suggested by the X-ray crystallographic structure of Perutz for horse hemoglobin (Perutz et al., 1968). The work on hemoglobin gives us some confidence regarding the validity of the use of these constants for probing the contact areas of subunit proteins.

The K_B values and the related K_s values employed in this study and the apparent number of amino acids at the contact areas of the subunits, N_{app}, required to fit the more reliable data are listed in Table II. The mean value of N_{app} based on the pH 9.5 data is 23 ± 4 whereas the value based on the pH 7.8 data in the presence of 0.01 M Ca^{2+} ion is somewhat higher, 24 ± 8. In the absence of Ca^{2+} at pH 7.8, the molecular weight of hemocyanin is found to be about (8.2–8.7) × 10^5 (Figure 6), suggesting a significant degree of dissociation. The addition of calcium ion or any of the salts initially stabilize the dodecameric form of the hemocyanin, followed by destabilization or dissociation to hexamers. Correspondingly, the molecular weight shows an initial increase as the concentration of salt is increased from 0 to about 0.4–0.6 M, followed by a decrease in molecular weights at higher salt concentration (see Figure 6). At the higher salt concentrations, the molecular weights are found to be nearly the same as those obtained in the presence of 0.01 M Ca^{2+}. Because of these complications, we have not attempted to analyze the salt dissociation data obtained in the absence of calcium ion. It is significant that this initial stabilization of hemocyanin was not observed with the ureas that are typical nonelectrolytes (Figure 5).

Despite our finding that the dissociation behavior of *Homarus* hemocyanin in the presence of calcium ion is adequately described by our main expression 4 the effects of a moderate degree of molecular microheterogeneity on the N_{app} and K_W parameters extracted from the molecular weight data should not be discounted or ruled out entirely. Consequently, it is instructive to examine what effect a 10- or even a 100-fold change on the dissociation constant, K_w, would have on the derived N_{app} estimates listed in Table II. On the basis of eq 5, we find that the changes in $\Delta G°_w$ of ±1.4 to ±2.8 kcal mol^{-1} M^{-1} corresponding to such changes in K_w would alter the slopes of $\Delta G°_D$ vs. [D] plots by approximately 15–30%. Using the pH 9.5 data obtained with urea and $NaClO_4$ (Figure 7), we estimate that these changes in slopes correspond to N_{app} changes of about ±3 to 4 and ±7 to 8 amino acid groups. Since the effects of microheterogeneity will tend to broaden the molecular weight transitions, we would expect that the N_{app} derived from such data will be lowered. This could lead to an underestimate of the actual number of amino acids at the contact areas of the *Homarus* hemocyanin hexamers by about 3–8 groups. Thus, our N_{app} estimates of Table II should be viewed as a minimum estimate of the contact groups. At the present state of our understanding, it is apparent that both the structural significance of our N_{app} estimates and the largely unknown effects of molecular microheterogeneity on the estimates of the contact groups at the subunit interfaces of the hemocyanins will have to be fully explored.

References

Aune, K. C., & Tanford, C. (1969) *Biochemistry 8*, 4586–4590.

Bijholt, M. C. A., Van Bruggen, E. F. J., & Bonaventura, J. (1979) *Eur. J. Biochem. 95*, 399–405.

Chincholi, B. S., Havlik, A. J., & Vold, R. D. (1974) *J. Chem. Eng. Data 19*, 148–152.

Di Giamberardino, L. (1967) *Arch. Biochem. Biophys. 118*, 273–278.

Eisenberg, H., Josephs, R., Reisler, J., & Schellman, J. A. (1977) *Biopolymers 16*, 2733–2783.

Elbaum, D., & Herskovits, T. T. (1974) *Biochemistry 13*, 1268–1278.

Ellerton, D. H., Carpenter, D. E., & Van Holde, K. E. (1970) *Biochemistry 9*, 2225–2232.

Herskovits, T. T., & Ibanez, V. S. (1976) *Biochemistry 15*, 5715–5721.

Herskovits, T. T., Gadegbeku, B., & Jaillet, H. (1970) *J. Biol. Chem. 245*, 2588–2598.

Herskovits, T. T., Cavanagh, S. M., & San George, R. C. (1977) *Biochemistry 16*, 5795–5801.

Herskovits, T. T., San George, R. C., & Cavanagh, S. M. (1978) *J. Colloid Interface Sci. 63*, 226–234.

Herskovits, T. T., Erhunmwunsee, L. J., & San George, R. C. (1981) *Biochim. Biophys. Acta 667*, 44–58.

Jeffrey, P. D. (1979) *Biochemistry 18*, 2509–2513.

Jolly, D., & Eisenberg, H. (1976) *Biopolymers 15*, 61–95.

Kaye, W., & Havlik, A. J. (1973) *Appl. Opt. 12*, 541–550.

Klarman, A., Gottlieb, J., & Daniel, E. (1979) *Biochemistry 18*, 2239–2244.

Konings, W. N., Siezen, R. J., & Gruber, M. (1969) *Biochim. Biophys. Acta 194*, 376–385.

Kruis, A. (1936) *Z. Phys. Chem. Abt. B 34B*, 13–50.

Kuiper, H. A., Gaastra, W., Beintema, J. J., van Bruggen, E. F. J., Schepman, M., & Drenth, J. (1975) *J. Mol. Biol. 99*, 619–629.

Morimoto, K., & Kegeles, G. (1971) *Arch. Biochem. Biophys. 142*, 247–257.

Nandi, P. K., & Robinson, D. R. (1972a) *J. Am. Chem. Soc. 94*, 1299–1308.

Nandi, P. K., & Robinson, D. R. (1972b) *J. Am. Chem. Soc. 94*, 1308–1315.

Perlmann, G. E., & Longsworth, L. G. (1948) *J. Am. Chem. Soc. 70*, 2719–2724.

Perutz, M. F. (1978) *Science (Washington, D.C) 201*, 1187–1191.

Perutz, M. F., Muirhead, H., Cox, J. M., & Goaman, L. C. G. (1968) *Nature (London) 219*, 131–139.

Robinson, D. R., & Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 2470–2479.

Robinson, R. A., & Stokes, R. H. (1959) in *Electrolyte Solutions*, pp 492–493, Butterworth, London.
Roxby, R., Miller, K., Blair, D. P., & Van Holde, K. E. (1974) *Biochemistry 13*, 1662–1668.
Scatchard, G., Hammer, W. J., & Wood, S. E. (1938) *J. Am. Chem. Soc. 60*, 3061–3070.
Schrier, E. E., & Schrier, E. B. (1967) *J. Phys. Chem. 71*, 1851–1860.
Schroder, E., Wollmer, A., Kubicki, J., & Ohlenbusch, H. D. (1976) *Biochemistry 15*, 5693–5697.
Siezen, R., & Van Driel, R. (1973) *Biochim. Biophys. Acta 295*, 131–139.
St. Pierre, T., & Jencks, W. P. (1969) *Arch. Biochem. Biophys. 133*, 99–102.
Tanford, C. (1969) *J. Mol. Biol. 39*, 539–544.
Van Bruggen, E. F. J., Schuiten, V., Wiebenga, E. H., & Gruber, M. (1963) *J. Mol. Biol. 7*, 249–253.
Van Holde, K. E., Blair, D., Eldren, N., & Arisaka, F. (1977) in *Structure and Function of Hemocyanin* (Bannister, J. V., Ed.) pp 22–30, Springer-Verlag, West Berlin.
von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 457–574, Marcel Dekker, New York.

Pressure-Induced Reversible Dissociation of Enolase†

Alejandro A. Paladini, Jr.,‡ and Gregorio Weber*

ABSTRACT: A study of the polarization of the intrinsic fluorescence and the fluorescence of dansyl conjugates of enolase shows that an increase in hydrostatic pressure, in the range of 1 bar–3 kbar, promotes the dissociation of this protein into dimers. The dissociation of oligomeric proteins under pressure is predicted to be a general phenomenon by a model that assumes the existence of small "free volumes" at the intersubunit boundaries. The same model predicts a dependence of the standard volume change in the dissociation reaction upon the pressure, owing to the additional surface compressibility of the monomers, and numerical analysis of the results clearly shows that dependence for enolase. For a midpoint dissociation pressure of 1.5 kbar the standard volume change in the dissociation reaction is $\Delta V_p^0 = -65 \pm 8$ mL mol^{-1}, and the dependence of the volume change upon pressure (dV_p^0/dp) is approximately −30 mL mol^{-1} kbar^{-1}. The reversibility of the pressure effects is shown to be better than 95% by either polarization or fluorescence spectrum recovery. The pressure perturbation of the fluorescence polarization is a method of general applicability to studies of protein aggregation, and it can be also of value in characterizing the effect of ligands on the aggregation of oligomeric proteins.

In the last few years we have conducted a series of studies both on single-chain proteins (Li et al., 1976a,b; Visser et al., 1977b) and on suitable molecular complexes (Weber et al., 1974; Visser et al., 1977a; Torgerson et al., 1979) to ascertain the possible causes of the changes in equilibrium conformation of proteins under high hydrostatic pressures, in the range of 10^{-3}–12 kbar. These investigations have pointed definitely to the primary importance of the covalent bond architecture in determining both the *sign and magnitude* of the characteristic equilibrium changes under pressure. Following their experiments with the inclusion complexes of cyclodextrin polymers, Torgerson et al. (1979) proposed that binding sites be classified as "soft" or "hard" according to whether they can or cannot, respectively, reduce their volume under pressure. This reduction of size can only be achieved by rotation about covalent bonds since both bond angles and bond lengths remain unchanged over the range of pressures at which observations in liquid water solution are possible, 12 kbar. The constancy of bond angles and bond lengths will, in general, restrict the approach of amino acid residues to each other toward the distances that would minimize their free energy of interaction in the absence of constraints and will lead to the appearance of small "free volumes" or "dead spaces" in the interior of a peptide chain when this adopts a globular folded conformation. Dilatometric studies (Zamyatnin, 1972) show that an increase in volume on the order of 2% should attend the folding of a peptide chain to the globular form, and analysis of X-ray crystallographic data of Richards (1977) directly validates the concept of small free volumes distributed throughout the protein structure. The existence of these small free volumes gives also an explanation of a finding of Karplus and coworkers in their study of the molecular dynamics of proteins by computer simulation: the appearance of very fast (picosecond) low-amplitude motions of the amino acid residues, faster in fact than those that would be expected in a homogeneous liquid.

Viewed from this standpoint the oligomeric proteins present a particularly interesting case: The contact areas between subunits with their multiple points of contact, determined by the folding of each peptide chain into a compact globule, must be considered as typical hard binding sites. We cannot expect that the subunit faces will make perfect van der Waals contacts throughout. Rather minimal distances and optimal atomic packing will prevail at those contacts, probably a small minority, which are the main origin of the free energy of subunit association while at other points it will be on average distinctly larger than the interatomic distances between nearby molecules of a liquid. The small free volumes so created will disappear upon dissociation owing to the much better packing of molecules of the liquid solvent against each subunit interface than the packing of these against each other. Accordingly, the existence of these dead spaces dictates that dissociation of the oligomer into subunits will proceed with decrease in volume ($\Delta V_{diss}^0 < 0$). A few observations in the literature indicate that this may well be the general case (Salmon, 1975; En-

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received September 19, 1980.* This work was supported by National Institutes of Health Grant GM 11223. A.R.P. was partly supported during this work by a fellowship from the Organization of American States.

‡ Present address: Instituto de Investigaciones Bioquimicas, Obligado 2490, 1429 Buenos Aires, Argentina.

0006-2960/81/0420-2587$01.25/0