Esaki, N., Suzuki, T., Tanaka, H., Soda, K., & Rando, R. R. (1977) *FEBS Lett. 84*, 309.
Esaki, N., Tanaka, H., Uemura, S., Suzuki, T., & Soda, K. (1979) *Biochemistry 18*, 407-410.
Golichowski, A., Harruff, R. C., & Jenkins, W. T. (1977) *Arch. Biochem. Biophys. 178*, 459-467.
Guggenheim, S., & Flavin, M. (1969) *J. Biol. Chem. 244*, 6217—6227.
Johnston, M., Marcotte, P., Donovan, J., & Walsh, C. (1979a) *Biochemistry 18*, 1729-1738.
Johnston, M., Jankowski, D., Marcotte, P., Tanaka, H., Esaki, N., Soda, K., & Walsh, C. (1979b) *Biochemistry 18*, 4690-4701.
Johnston, M., Raines, R., Walsh, C., & Firestone, R. A. (1980) *J. Am. Chem. Soc. 102*, 4241-4250.
Kainosho, M., Ajisaka, K., Kamisaku, M., & Murai, A. (1975) *Biochem. Biophys. Res. Commun. 64*, 425-432.
Lavine, T. F. (1947) *J. Biol. Chem. 169*, 477.
Posner, B. I., & Flavin, M. (1972a) *J. Biol. Chem. 247*, 6402-6411.
Posner, B. I., & Flavin, M. (1972b) *J. Biol. Chem. 247*, 6412-6419.
Soda, K. (1968) *Anal. Biochem. 25*, 228.
Soda, K., & Osumi, T. (1971) *Methods Enzymol. 17B*, 629-636.
Soda, K., Tochikura, T., & Katagiri, H. (1961) *Agric. Biol. Chem. 25*, 811.
Tanaka, H., Esaki, N., Yamamoto, T., & Soda, K. (1976) *FEBS Lett. 66*, 307.
Tanaka, H., Esaki, N., & Soda, K. (1977) *Biochemistry 16*, 100.
Toennis, G., & Kolb, J. J. (1941) *J. Biol. Chem. 140*, 131.
Walsh, C. (1979) in *Enzymatic Reaction Mechanism*, pp 777-827, W. H. Freeman, San Francisco.
Washtien, W., Cooper, A. J. L., & Abeles, R. H. (1977) *Biochemistry 16*, 460-463.

Light-Scattering Investigation of the Dissociation Behavior of *Lunatia heros* and *Littorina littorea* Hemocyanins†

Theodore T. Herskovits* and L. John Mazzella
Department of Chemistry, Fordham University, Bronx, New York 10458
German B. Villanueva
Department of Biochemistry, New York Medical College, Valhalla, New York 10595
Received November 27, 1984

ABSTRACT: The subunit structure and dissociation of the hemocyanins of two marine snails, *Lunatia heros* and *Littorina littorea*, were investigated by light-scattering molecular weight methods. The hemocyanins of both species of snails are readily dissociated to fragments of one-tenth and one-twentieth of the parent proteins of close to 9×10^6 daltons by either increasing the pH or using dissociating reagents of the hydrophobic urea series or some of the Hofmeister salts. The lower members of the latter group of reagents, NaCl, and to some extent also NaBr were found to have only marginal effects on the observed molecular weight transitions, suggesting that the two hemocyanins investigated possess β-type subunits, which are known to be resistant to NaCl dissociation. The molecular weight profiles obtained with the various dissociating reagents were single inverted sigmoidal-shaped curves for both *Lunatia* and *Littorina* hemocyanins, suggesting overlapping transitions. The ultracentrifugation patterns and the species-distribution plots based on the urea dissociation data of *Littorina* hemocyanin suggest the presence of whole, half, and one-tenth molecular weight species in the dissociation transition region. Fitting of the urea dissociation data of *Littorina* hemocyanin obtained at both pH 5.7 and pH 8.0, assuming a sequential two-step dissociation scheme used in our previous studies [Herskovits, T. T., & Russell, M. W. (1984) *Biochemistry 23*, 2812-2819], was found to be consistent with a model of a few hydrophobic binding sites at the contact areas of the half-molecules and a much larger apparent number of binding sites (N_{app}) at the side to side contacts of the one-tenth molecules. Model calculations also showed that with much larger N_{app} values for the first dissociation step, approaching that of the second step of the dissociation reaction (N_{app} = 50-60), biphasic curves would be produced. This suggests that the stabilizing interactions between the basic decameric units of the marine hemocyanins are largely nonhydrophobic in origin and therefore must be due primarily to polar and ionic interactions. The much larger N_{app} estimates characterizing the second step of the dissociation reaction are comparable to those obtained with the α component of *Helix pomatia* hemocyanin. Hydrophobic stabilization of each half-molecule through side to side contact of the dimeric subunits is suggested by both the latter observations and also our finding that the dissociation of *Littorina*, as well as *Lunatia*, hemocyanin by the urea series follows the expected order of increasing effectiveness with increasing hydrophobicity of the dissociating reagent.

The hemocyanins are copper-containing multisubunit proteins that serve as oxygen carriers in the circulatory systems of many arthropods and molluscs. The hemocyanins of the latter phylum are large cylindrical particles of decameric assemblies consisting usually of one or two such assemblies. The hemocyanins of octopi and squids are the simpler single-assembly particles while the land and marine snail hemocyanins are the

† Supported in part by Grant RR-0715 from the National Institutes of Health, U.S. Public Health Service.

0006-2960/85/0424-3862$01.50/0

larger two-assembly proteins (Bonaventura et al., 1977; Van Holde & Miller, 1982; Ellerton et al., 1983). With some gastropod species, such as the sea hare, *Aplysia limacina* (Ghiretti-Magaldi et al., 1979), the keyhole limpet, *Megathura crenulata* (Senozan et al., 1981), or the two marine snails of this study, *Littorina littorea* (Svedberg & Pedersen, 1940) and *Lunatia heros*, trimeric and higher oligomeric assemblies of hemocyanin particles have also been observed, especially at higher protein concentrations. Electron microscopic studies suggest cylindrical particles of about 300 Å in diameter and 340–380 Å high and linear stacks or aggregates of such particles (Van Bruggen et al., 1962; Condie & Langer, 1964; Ghiretti-Magaldi et al., 1979). The hemocyanins of the cephalopods, the squids, octopi, and nautili, have about the same diameter but only about half the height and molecular mass as the snail hemocyanins. The reported molecular masses of the gastropod hemocyanins are in the range of (7.5–9) × 10^6 daltons (Wood et al., 1971; Van Holde & Miller, 1982; Herskovits & Russell, 1984).

In recent years a substantial number of investigations have addressed various aspects of the problem of subunit organization and structure–function relationships of the hemocyanins of arthropods and molluscs [for more recent reviews, see Van Holde & Miller (1982) and Ellerton et al. (1983)]. However, the problem of forces that stabilize the quaternary structure and hold the subunits together in solution has gained comparatively little attention. In the cases of two of the arthropod hemocyanins, the proteins of the lobster, *Homarus americanus*, and the blue crab, *Callinectes sapidus*, investigated in our laboratory, molecular weight studies with various ureas and salts as dissociating agents have suggested that the contact areas between the two basic hexameric units as well as the individual subunits in the hexamers are stabilized by polar and ionic interactions (Herskovits et al., 1981a,b). With the molluscan hemocyanins, an entirely different pattern of subunit stabilization is emerging, on the basis of our initial work with the α component of *Helix pomatia* hemocyanin (Herskovits & Russell, 1984). With this component of land snails, which is readily dissociated by salts, the contact areas between the two basic cylindrical units appear to be largely nonhydrophobic in nature. However, the side to side contacts between the five dimeric subunits, which constitute in solution each of the half-molecules, are hydrophobically stabilized.

In the present study, we have extended our investigations to the β-type hemocyanins (Wood, 1973) of two marine snails. This more prevalent molluscan form of hemocyanin is not dissociated by 1.0 M NaCl to half-molecules (Brohult, 1947; Heirwegh et al., 1961) and appears to be also more resistant to dissociation by the other neutral salts of the Hofmeister series. We find, however, that both *Lunatia heros* and *Littorina littorea* hemocyanins are effectively dissociated by the hydrophobic urea series of reagents proposed earlier as probes for the contact areas of subunit proteins in solution (Herskovits et al., 1977, 1978).

Materials and Methods

Live specimens of *Lunatia heros* snails were obtained from the Marine Biological Laboratory of Woods Hole, MA. The hemolymph was collected from deep incisions made in the exposed foot of the snails with the shells inverted over beakers containing 1–2 mL of freshly prepared phenylmethanesulfonyl fluoride solutions, dissolved in cold 0.1 M pH 5.7 sodium acetate, containing also some undissolved crystals of the protease inhibitor. In order to facilitate the collection of the blood, some of the shell protecting the foot area of the individual animals were fractured and removed, care being exercised so as to avoid injury to any of the soft neighboring tissue. Live *Littorina littorea* snails were purchased from Randazzo's Fish Market, Bronx, NY. Due to the relatively small size of these snails, the hemolymph was collected from the edges of the aperture region of the inverted animals, following thorough washing and removal of the operculi by excision. Small syringes containing about 0.2 mL of freshly prepared phenylmethanesulfonyl fluoride suspension, in pH 5.7 acetate buffer, were used to collect the hemolymph, per dozen or so snails processed in each batch. The pooled hemolymph of both species of snails was freed of cell debris by centrifugation at 6000*g* for 15 min in the cold and purified by gel filtration on 35 × 4.0 or 45 × 2.5 cm Bio-Gel A-5m (200–400 mesh) columns equilibrated in the cold with 0.1 M pH 5.7 acetate as previously described (Herskovits et al., 1981a,b). With most of the work on *Littorina* hemocyanin, 0.01 M Mg^{2+} and 0.01 M Ca^{2+} was also included in the acetate buffer used as eluent. The hemolymph of *Lunatia* snails was found to have a relatively low hemocyanin concentration, ranging from about 0.3% to 0.8%, and a pH of 8.1–8.2. The concentrations of hemocyanin in the hemolymph of *Littorina* snails were found to be appreciably higher, ranging from 1.1% to 3.3%. The pH of the hemolymph of the latter snails was 7.8–8.2

Protein concentrations were determined spectrophotometrically on a Cary 14 recording instrument with extinction coefficients E^{1cm}_{278} of 16.76 for *Lunatia* hemocyanin and of 16.70 for *Littorina* hemocyanin at pH 5.7. These extinction values were based on specific refractive index determinations made on solutions using the $(\partial n/\partial c)_\mu$ value of 0.194 g^{-1} cm^3, obtained for *Busycon canaliculatum* hemocyanin (Herskovits et al., 1985). This value of $(\partial n/\partial c)_\mu$ is within ±0.003 g^{-1} cm^3 or about 2% of the values of this parameter reported for other molluscan hemocyanins, such as that of the squid, *Loligo paelei* (Van Holde & Cohen, 1964), and the snail, *Helix pomatia* and *B. canaliculatum* (Herskovits et al., 1984, 1985). The extinction coefficients in dissociating and denaturing solvents were based on absorbance determinations made on solutions diluted from common stock solutions, using the pH 5.7 extinction coefficients as reference.

Light-scattering and specific refractive index increment measurements were made at 436 nm in a Wood Manufacturing Co. instrument of Brice's design, which measures absolute turbidities. The solutions and solvents used for light-scattering measurements were filtered directly into the light-scattering cells, using single 25-mm 0.20-mμ Gelman membrane filters mounted in Millipore filter holders (Harrington et al., 1973; Elbaum & Herskovits, 1974). The light-scattering data measured at an angle of 90° were interpreted by means of the familiar equation

$$K'c/R_\theta = 1/M_w + 2B'c \qquad (1)$$

where R_θ represents the excess Rayleigh ratio of the protein solution minus that of the solvent, c is the protein concentration, M_w is the weight-average molecular weight, B' is the second virial coefficient, and K' is the light-scattering constant (Doty & Edsall, 1951). Protein solutions of the same concentration containing increasing amounts of urea or other dissociating reagent used for light scattering as well as other measurements were prepared by serial dilution from common stock solutions of protein and more concentrated reagent. The specific refractive index increment $(\partial n/\partial c)_\mu$ in aqueous solutions, 8.0 M urea, and 6.0 M GdmCl[1] solvents was determined

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; GdmCl, guanidinium chloride; CD, circular dichroism.

Table I: Light-Scattering Molecular Weight Data of *Lunatia heros* and *Littorina littorea* Hemocyanin

solvent	protein concn range (g L^{-1})	$(\partial n/\partial c)_\mu$ (g^{-1} cm^3)	M_w	B' (L mol g^{-2})
		Lunatia heros Hc		
pH 7.6, μ = 0.1 phosphate	0.05–1.6	0.194[a]	(8.9–11.8) × 10^6[b]	
pH 5.7, μ = 0.1 acetate	0.5–2.9	0.194	(9.2–13.4) × 10^6[b]	
1.0 M NaCl, μ = 0.1 pH 7.6 phosphate	0.1–0.9	0.186[a]	7.97 × 10^6	−1 × 10^{-8}
pH 10.6, μ = 0.1 bicarbonate–NaOH	0.2–0.8	0.205[a]	4.46 × 10^5	−4 × 10^{-7}
8.0 M urea, μ = 0.1 pH 7.6 phosphate	0.2–0.9	0.139	4.79 × 10^5	3 × 10^{-7}
6.0 M GdmCl, pH 5.7 acetate	0.5–1.0	0.135	3.35 × 10^5	3 × 10^{-7}
		Litorina littorea Hc		
pH 5.7, μ = 0.1 acetate	0.25–1.8	0.194	(8–11) × 10^6[b]	
pH 10, μ = 0.1 bicarbonate–NaOH	0.26–1.8	0.193	4.63 × 10^5	−1 × 10^{-7}
8.0 M urea, μ = 0.1 pH 8.2 phosphate	0.26–1.3	0.136	4.72 × 10^5	3 × 10^{-7}
6.0 M GdmCl, μ = 0.1 pH 5.7 acetate	0.24–1.1	0.140	2.87 × 10^5	−1 × 10^{-7}

[a] Assumed values based on *Busycon canaliculatum* hemocyanin data (Herskovits et al., 1985). [b] The range of values given for *Lunatia* and *Littorina* hemocyanin at pH 7.6 and 5.7 is for the protein concentration ranges specified in column 2 (see text). The rest of the molecular weight data represent extrapolated values to infinite dilution, based on eq 1.

on dialyzed solutions as previously described (Elbaum & Herskovits, 1974). For the various urea and salt solutions, the secondary influence of reagent on $(\partial n/\partial c)_\mu$ was estimated by employing the same molar decrement of 0.004–0.010 g^{-1} cm^3 as in our previous studies on *H. pomatia* and *B. canaliculatum* hemocyanin (Herskovits et al., 1984, 1985).

Sedimentation velocity measurements were made in a Beckman Model E analytical ultracentrifuge using schlieren optics. Viscosity and density corrections required for the urea data were based on interpolation of the values given in the *Handbook of Chemistry and Physics* (1979).

Circular dichroism measurements were made on a Cary 60 spectropolarimeter using 1.0- and 0.10-cm cylindrical cells. Mean residue molecular weights of 122 based on the amino acid data of Dijk et al. (1970) were used for all our calculations of the mean residue ellipticities. The molar ellipticities in the copper absorbance region were based on an assumed molecular mass of 2.5 × 10^4 daltons per copper atom.

RESULTS

Concentration Dependence of the Light-Scattering Data. The light-scattering behavior of *Lunatia* and *Littorina* hemocyanins investigated in both aqueous media and dissociating and denaturing solvents is shown in Figure 1. The summary of the data of Table I suggests molecular masses of (4.4–4.8) × 10^5 daltons for the fully dissociated subunits at high pH and in the presence of 8.0 M urea. These molecular weights are close to one-twentieth of the molecular weights of the didecameric assemblies of hemocyanin found in the hemolymph of many marine snails (Wood et al., 1971; Van Holde & Miller, 1982). In both concentrated urea solutions and at high pH, the hemocyanins retain much of their secondary structure as suggested by their CD and absorbance spectra. The drop in molecular masses in 6.0 M GdmCl much below 4.5 × 10^5 daltons seem to reveal some of the hidden breaks in the polypeptide chains of hemocyanin, which are known to be very susceptible to proteolysis (Gielens et al., 1975; Brouwer et al., 1978). The extrapolated values of the molecular masses close to 9 × 10^6 daltons obtained with *Lunatia* hemocyanin are encouraging but require critical comments and further study. The ultracentrifugation behavior examined at higher concentration close to 3 g L^{-1} suggests largely 130S particles[2] assembled of three basic decameric units (Figure 2A), each

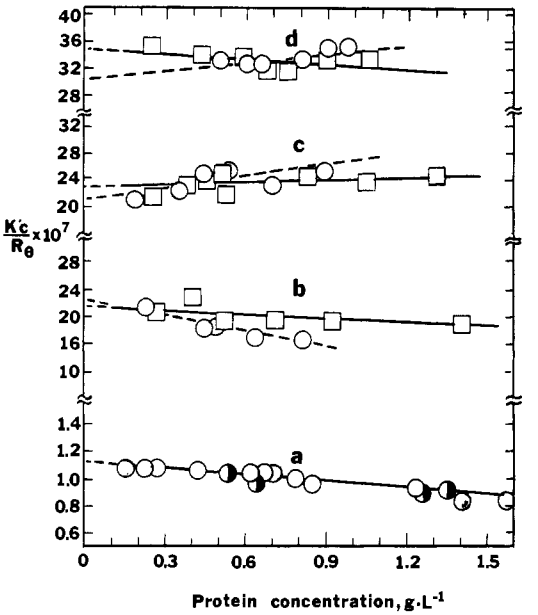

FIGURE 1: Concentration dependence of the light-scattering of *Lunatia heros* (circles) and *Littorina littorea* (squares) hemocyanins plotted according to eq 1: (a) *Lunatia heros* hemocyanin, (○) pH 5.7, μ = 0.1 acetate, (◑) pH 7.6, μ = 0.1 phosphate; (b) *Lunatia* Hc (○), pH 10.6, μ = 0.1 bicarbonate–NaOH; *Littorina* Hc (□), pH 10, μ = 0.1 bicarbonate–NaOH; (c) 8.0 M urea, *Lunatia* Hc (○), pH 7.6 phosphate; *Littorina* Hc (□), pH 8.2, μ = 0.1 Tris; (d) 6.0 M GdmCl, *Lunatia* Hc (○), pH 5.7 acetate; *Littorina* Hc (□), pH 5.7 acetate.

with molecular masses close to 4.5 × 10^6 daltons. This is also suggested by the light-scattering data obtained at corresponding concentrations shown in Figure 1 (curve a). The negative slope of the concentration dependence of the light-scattering plot suggests dissociation and reequilibration to largely dimeric assemblies as the protein concentration is reduced. Thus, the apparent molecular masses, which have lead to the extrapolated value of (8.88 ± 0.07) × 10^6 daltons at pH 7.6 (corresponding to infinite dilution based on eq 1), were found to be 9.27 × 10^6, 10.1 × 10^6, 10.7 × 10^6, and 11.8 × 10^6 at protein concentrations of 0.28, 0.70, 1.24, and 1.59 g L^{-1}, respectively. The upper values of the apparent molecular masses approach the expected value of (13.2–13.5) × 10^6 daltons for the trimeric assembly of hemocyanin components suggested by the ultracentrifugation data at somewhat higher protein concentrations.

The tendency of certain species of hemocyanin to form higher polymers or aggregates of assemblies has recently been discussed by Ghiretti-Magaldi et al. (1979) and Van Holde & Miller (1982) in regard to the influence of hydrostatic

[2] For the hemocyanin components usually designated as 130S, 100S, and 60S particles (Van Holde & Miller, 1982), our observed values at protein concentrations of 3–6 g L^{-1} were found to be 135–138 S, 93–106 S, and 66–68 S, respectively.

Table II: Effects of pH, Urea, and GdmCl on the Macromolecular and Spectroscopic Parameters of *Littorina littorea* and *Lunatia heros* Hemocyanin

solvent	M_w	dL g^{-1} cm^{-1} ϵ_{278}	dL g^{-1} cm^{-1} ϵ_{346}	deg cm^2 dmol^{-1} $[\theta]_{222}$	deg cm^2 dmol^{-1} $[\theta]_{M,346}$
	Littorina littorea Hc				
pH 5.7, μ = 0.1 acetate, 0.01 M Mg^{2+}, 0.01 M Ca^{2+}	(8–11) × 10^6 [a]	16.7	4.8	−6900 ± 600	−29 500
pH 8.0, 0.01 M Mg^{2+}	6.0 × 10^6		4.50	−6900	−34 800
pH 8.4, μ = 0.1 Tris	6.7 × 10^5		3.93	−6800	−40 900
pH 10, μ = 0.1 bicarbonate–NaOH	4.6 × 10^5	14.1	3.34	−6600	−38 200
8.0 M urea, pH 8.2 Tris	4.4 × 10^5	13.5	2.75	−4900	−26 700
6.0 M GdmCl, pH 5.7 acetate	2.9 × 10^5	14.4	0	−1000	0
	Lunatia heros Hc				
pH 7.6, μ = 0.1 phosphate	(8.9–11.8) × 10^6 [a]	16.8	4.8	−6100	−31 600
pH 8.2, μ = 0.1 Tris	6.4 × 10^5		3.4		
pH 10.6, μ = 0.1 bicarbonate–NaOH	4.5 × 10^5	13.3	3.2		
8.0 M urea, pH 7.6 phosphate	4.8 × 10^5	12.9	3.1	−4300	−29 500
6.0 M GdmCl, pH 7.6 phosphate	3.5 × 10^5	13.2	~0	~−500	~0

[a] Apparent molecular weight values obtained in the protein concentration range of 0.05–1.8 g L^{-1} (see text).

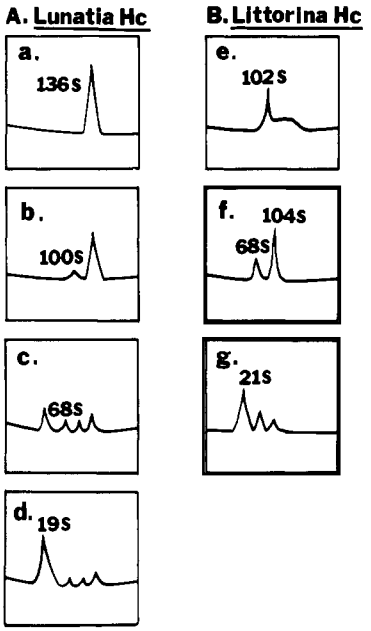

FIGURE 2: Tracings of the ultracentrifugation patterns of *Lunatia heros* and *Littorina littorea* hemocyanins. (A) *Lunatia* Hc, pH 7.6, μ = 0.1 phosphate; (a) 0 M urea; (b) 1 M urea; (c) 2 M urea; (d) 3 M urea. (B) *Littorina* Hc: (e) pH 5.7, μ = 0.1 acetate; (f) pH 8.0, μ = 0.1 Tris, 0.01 M Mg^{2+}; (g) 1.5 M urea, pH 8.0 Tris, 0.01 M Mg^{2+}. Rotor speed was 24 630 rpm; temperature was 20 °C; protein concentration was 3–6 g L^{-1}.

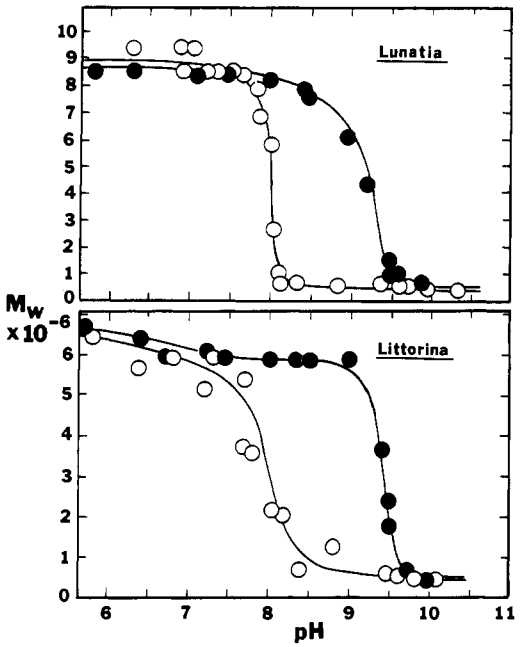

FIGURE 3: pH dependence of the molecular weight behavior of *Lunatia heros* and *Littorina littorea* hemocyanin studied in the absence (open circles) and the presence of 0.01 M Mg^{2+} (filled circles). Protein concentrations employed were 5 × 10^{-2} and 25 × 10^{-2} g L^{-1}. The 0.1 ionic strength buffers used were as follows: acetate, pH 5.5–5.7; phosphate, pH 6.2–8.0; Tris, pH 8.0–9.7; bicarbonate–NaOH, pH 9.8–10.6.

pressure in the ultracentrifuge on the stability of such aggregates, observed in electron microscopy. In light scattering, aggregation produces negatively sloping $K'c/R_\theta$ vs. concentration plots and also negative second virial coefficients. Interestingly, *Littorina* hemocyanin, which shows a greater propensity for dissociation at low protein concentration, has an apparent negative slope of about 3 times that of *Lunatia* hemocyanin. The extrapolated value of the molecular mass at infinite dilution obtained at pH 5.7 is (7.0 ± 0.1) × 10^6 daltons, suggesting significant amount of dissociation at low hemocyanin concentrations. The apparent molecular masses of both hemocyanins reach about the same value of close to 12 × 10^6 daltons at protein concentrations approaching 2 g L^{-1}.

pH Dependence of the Light-Scattering Data. Figure 3 shows the molecular weight behavior of both *Lunatia* and *Littorina* hemocyanins studied as a function of pH. The pH dependence shows a relatively sharp drop in molecular weights over relatively narrow ranges of pH 7.8–8.2 for both proteins. We find no plateau region in the molecular weight transitions in either the presence or the absence of stabilizing Mg^{2+} ions, signifying the accumulation of a significant fraction of half-molecules or 60S particles, before further dissociation to one-tenth molecules occurs. For both proteins the pH dissociation of the one-tenth to one-twentieth particles is found to be much more gradual. For example, with *Lunatia* hemocyanin, molecular masses of (7.9–1.1) × 10^6 daltons were obtained at pH 7.8–8.1, with a subsequent drop to 6.4 × 10^5, 5.8 × 10^5, and 4.7 × 10^5 daltons at pH 8.2, 9.9, and 10.5, respectively (Figure 3). It should also be noted that neither pH nor urea dissociation produce extensive unfolding or denaturation in the hemocyanin subunits. This is suggested by the fact that both the copper absorbance, at 346 nm, and the CD spectra at this band and the lower lying α-helix band, at 222 nm, are largely preserved following exposure of both hemocyanins to alkaline pHs and 8 M urea (see Table II).

Subunit Dissociation by the Ureas and Hofmeister Salts. The subunit dissociation of both *Lunatia* and *Littorina* he-

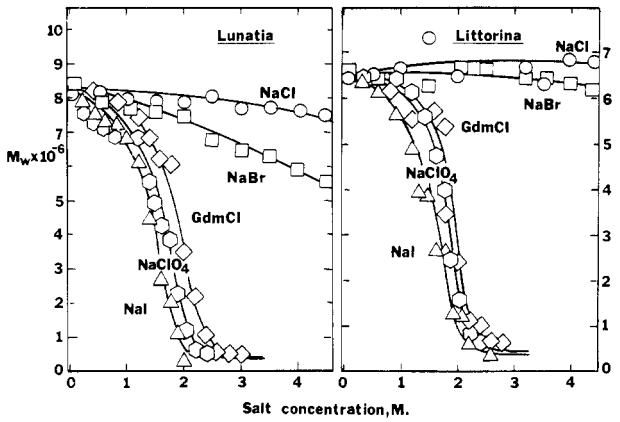

FIGURE 4: Effects of various salts and GdmCl on the subunit dissociation of *Lunatia heros* hemocyanin (pH 7.6) and *Littorina littorea* hemocyanin (pH 5.7) followed by molecular weight changes (M_w) as a function of salt concentration. Hemocyanin concentration was 5×10^{-2} g L^{-1}; buffers employed were the same as in Figure 3.

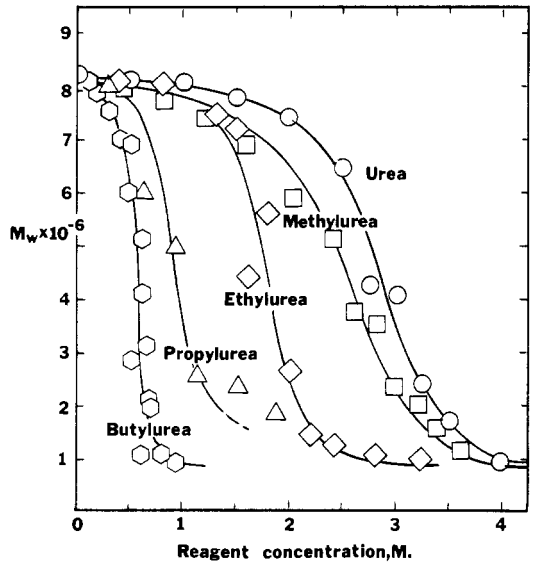

FIGURE 5: Effects of the hydrophobic urea series of reagents on the molecular weights (M_w) of *Lunatia heros* hemocyanin studied as a function of dissociating reagent concentration. Protein concentration was 5×10^{-2} g L^{-1}; buffer was $\mu = 0.1$ phosphate, pH 7.6.

mocyanins has been investigated with the urea series of dissociating agents as well as the Hofmeister salts to affect dissociation. Figures 4–6 present some of our light-scattering molecular weight data obtained at hemocyanin concentrations of 5×10^{-2} g L^{-1}. At these low protein concentrations the nonideality corrections due to second virial effects are very small and can thus be neglected. Aggregation occasionally observed at higher protein concentrations with some of the dissociating agents is also minimized at these concentrations (Herskovits et al., 1981, 1984).

Dissociation of both hemocyanins by the urea series shown in Figures 5 and 6 follows the trend of effectiveness expected of hydrophobically stabilized subunit systems. The salts NaCl and NaBr produce little or no dissociation expected of β-type hemocyanins (Heirwegh et al., 1961; Wood, 1973). It should be noted, however, that the higher, more effective members of the Hofmeister series, NaI and $NaClO_4$, can fully dissociate both *Lunatia* and *Littorina* hemocyanin (Figure 4).

In order to gain some further insight concerning the dissociation process, we have analyzed some of the *Littorina* data in terms of both the two-step dissociation scheme of whole–half–one-tenth molecules, and whole molecules dissociating

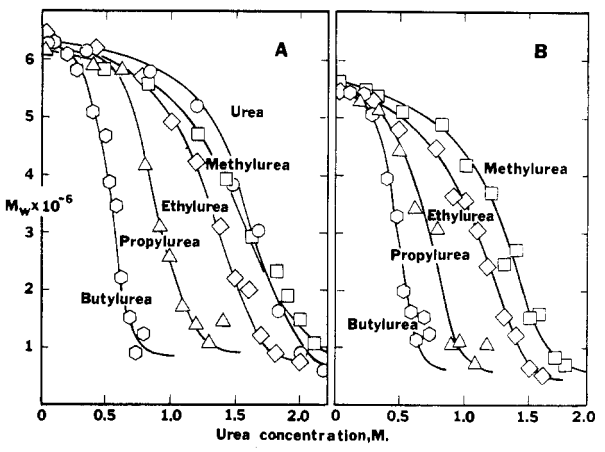

FIGURE 6: Effects on the urea series of reagents on the molecular weight (M_w) of *Littorina littorea* hemocyanin obtained at pH 8.0, $\mu = 0.1$ Tris, and 0.01 M Mg^{2+} (A) and in the presence of 1.2 M urea, pH 8.0, $\mu = 0.1$ Tris, and 0.01 M Mg^{2+} (B). Protein concentration was 5×10^{-2} g L^{-1}.

to one-tenth molecules. In terms of the weight fractions of whole molecules that dissociate to half-molecules, α_1, and half-molecules to dimers, α_2, the weight average molecular weight, M_w and the related expressions used to fit the experimental data have the form (Herskovits & Russell, 1984)

$$M_w = M_{20}(1 - 0.5\alpha_1 - 0.4\alpha_1\alpha_2) \quad (2)$$

and

$$\frac{\alpha_1^2}{1-\alpha_1} = \frac{K_{w,app}^{20,10} M_{20}}{4c(1-\alpha_2)^2} \exp(2N_{app}^{20,10} K_B c_D) \quad (3)$$

$$\frac{\alpha_2^5}{1-\alpha_2} = \frac{(2.0 \times 10^{-5}) M_{20}^4 K_{w,app}^{10,2}}{c^4 \alpha_1^4} \exp(5N_{app}^{10,2} K_B c_D) \quad (4)$$

For the one-step dissociation of whole molecules breaking up to one-tenth molecular weight fragments, the related expressions in terms of the weight fraction of dissociating hemocyanin, α_3, are

$$M_w = M_{20}(1 - 0.9\alpha_3) \quad (5)$$

and

$$\frac{\alpha_3^{10}}{1-\alpha_3} = \frac{(1.0 \times 10^{-10}) M_{20}^9 K_{w,app}^{20,2}}{c^9} \exp(10N_{app}^{20,2} K_B c_D) \quad (6)$$

In eq 2–6, $K_{D,app}$ and $K_{w,app}$ represent the apparent dissociation constants of hemocyanin in the presence and absence of dissociating reagent, N_{app} is the apparent number of amino acids at the contact areas of the subunits or dissociating fragments, specified by the superscripts for the different stages of dissociation, K_B is the binding or interaction constant of the dissociating agent with the average amino acid at the contact areas of the subunits, M_{20} is the molecular weight of undissociated whole hemocyanin, c is the concentration of hemocyanin in g L^{-1}, and c_D is the molar concentration of the dissociating reagent. Figures 7 and 8 show some of our data analyzed and fitted by using these equations.

The urea dissociation data of *Littorina* hemocyanin shown in Figures 7 and 8 could be analyzed by use of N_{app} and $K_{D,app}$ appropriate for either a single-step two-species transition or a two-step three-species dissociation transition (see curves a and c of Figure 7). The apparent anomaly suggested by the presence of more than two species of hemocyanin observed in the ultracentrifuge (Figure 2) could be rationalized by showing that we actually deal with overlapping two-step transitions,

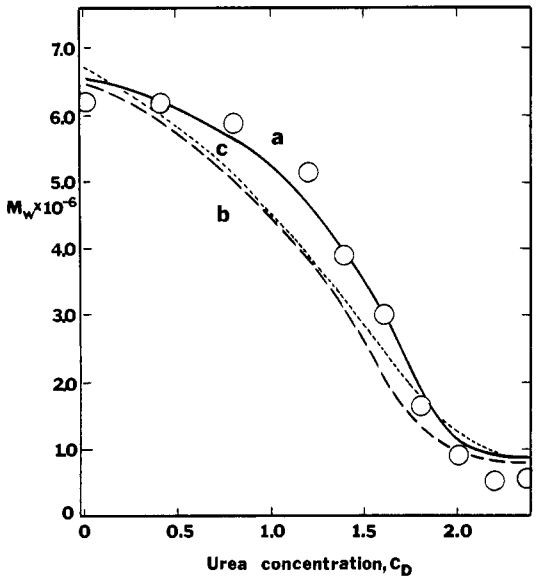

FIGURE 7: Fit of the urea dissociation data of *Littorina* hemocyanin at pH 8.0 (μ = 0.1 Tris, 0.01 M Mg^{2+}) based on eq 2–4 (curves a and b) and eq 5 and 6 (curve c) plotted as M_w vs. urea concentration, c_D. (Curve a) $N_{app}^{20,10} = 8$, $K_{w,app}^{20,10} = 1 \times 10^{-8}$ M, $N_{app}^{10,2} = 65$, and $K_{w,app}^{10,2} = 5 \times 10^{-37}$ M^4; (curve b) $N_{app}^{20,10} = 10$, $K_{w,app}^{20,10} = 1 \times 10^{-8}$ M, $N_{app}^{10,2} = 60$, and $K_{w,app}^{10,2} = 1 \times 10^{-35}$ M^4; (curve c) $N_{app}^{20,2} = 25$ and $K_{w,app}^{20,2} = 1 \times 10^{-71}$ M^9. With all the calculations, $M_{20} = 8.8 \times 10^6$, $K_B = 0.032$ M^{-1}, and $c = 5 \times 10^{-2}$ g L^{-1} were used.

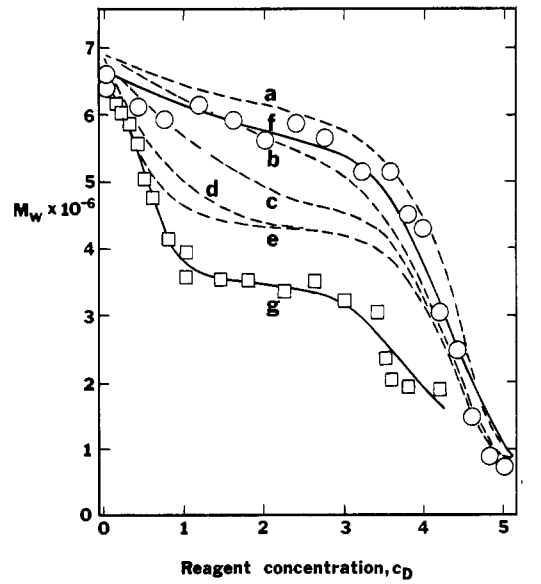

FIGURE 8: Resolution of the dissociation curves of hemocyanin with increasing N_{app} based on eq 2–4: (Curves a–e) $N_{app}^{20,10} = 5$, 10, 20, 40, and 60, all computed with the same $N_{app}^{10,2} = 60$, $K_{w,app}^{20,10} = 1 \times 10^{-8}$ M, $K_{w,app}^{10,2} = 1 \times 10^{-48}$ M^4, and $M_w = 8.8 \times 10^6$, $K_B = 0.032$ M^{-1}, and $c = 5 \times 10^{-2}$ g L^{-1}; (curve f) pH 5.7 urea data of *Littorina* hemocyanin fitted with $N_{app}^{20,10} = 8$, $K_{w,app}^{20,10} = 1.2 \times 10^{-8}$ M, $N_{app}^{10,2} = 60$, $K_{w,app}^{10,2}$ 1×10^{-48} M^4, and with the rest of the parameters same as above: (curve g) sodium acetate dissociation data of *Helix pomatia* α-hemocyanin at pH 5.7 taken from Herskovits & Russell (1984), fitted with $N_{app}^{20,10} = 155$, $K_{w,app}^{20,10} = 8.8 \times 10^{-10}$ M, $N_{app}^{10,2} = 63$, $K_{w,app}^{10,2} = 5.6 \times 10^{-41}$ M^4, $M_{20} = 7.2 \times 10^6$, $K_B = 0.021$ M^{-1}, and $c = 5 \times 10^{-2}$ g L^{-1}.

characterized by relatively low $N_{app}^{20,10}$ parameters and not overly different stabilization energies of the two steps of the dissociation reaction. The best fit of the urea dissociation data of Figure 8, represented by the circles and the solid curve f, was obtained with $N_{app}^{20,10} = 8$ and $N_{app}^{10,2} = 60$. The successive curves, a–e, of this figure represented by the dashed lines were calculated for increasing $N_{app}^{20,10}$ values ranging from 5 to 60

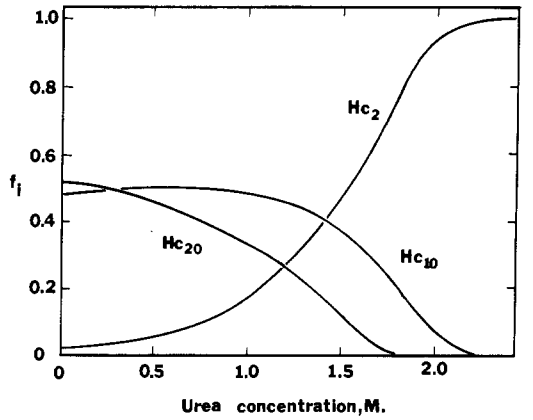

FIGURE 9: Species distribution plot of *Littorina* hemocyanin at pH 8.0 (0.01 M Mg^{2+}) obtained with urea as dissociating agent. The weight fraction of whole molecules (Hc_{20}), half-molecules (Hc_{10}), and one-tenth molecules (Hc_2) was calculated from the expressions $f_{20} = 1 - \alpha_1$, $f_{10} = \alpha_1 (1 - \alpha_2)$, and $f_2 = \alpha_1\alpha_2$, with the α_1 and α_2 values taken from the data set of curve a of Figure 7.

and a constant value of $N_{app}^{10,2} = 60$. It is apparent from these calculations that as the $N_{app}^{20,10}$ parameter of the first step of the dissociation reaction is increased, the observed single-phase transitions are resolved into the expected two-phase transitions. We have also calculated the species distributions of *Littorina* hemocyanin components, on the basis of the urea dissociation data obtained at pH 8.0 in 0.01 M Mg^{2+} containing solvent, shown in Figure 9.

The reassociation or reversibility of both *Lunatia* and *Littorina* hemocyanins was tested with urea as the dissociating agent. In these experiments, hemocyanin solutions [c = (2.5–5) $\times 10^{-2}$ g L^{-1}] were exposed for about 30 min to reagent concentrations where half-molecules and one-tenth molecules are the dominant dissociation species, followed by 2–4-fold dilutions with buffer. For both hemocyanins, a moderate degree of reassociation was noted from the largely half-molecular states, with much smaller extent of reassociation being observed for solvents where the hemocyanins are fully dissociated. For example, in the case of *Littorina* hemocyanin exposure to 4.0 M urea at pH 5.7, followed by dilution to 1.0 and 2.0 M urea, gave molecular masses of 4.2 $\times 10^6$ to 4.6 $\times 10^6$ daltons and about 1.0 $\times 10^6$ daltons after exposure to 5.0 M urea and dilution to 1.25 and 2.5 M urea. The pH 8.0 data of this hemocyanin diluted from 1.5 M urea gave nearly the same molecular masses of 4.1 $\times 10^6$ to 4.7 $\times 10^6$ daltons in 0.4–0.75 M urea. The species distribution of hemocyanin at this pH (Figure 9) suggests the presence of nearly 50% one-tenth molecules at 1.5 M urea. If we assume that essentially only half-molecules reassociate [the reassociation of one-tenth molecules appears to be much slower (Wood, 1973)], the molecular masses of the diluted solutions should have been close to 5 $\times 10^6$ daltons. This suggests close to 80% reassociation of the half-molecules of *Littorina* hemocyanin. Qualitatively similar reassociation data were obtained with *Lunatia* hemocyanin.

Circular Dichroism and Absorbance Studies. The data of Figures 10 and 11 and Table II represent some of spectrophotometric and molecular weight results. The mean residue ellipticities of −6100 ± 600 to −6900 ± 600 deg cm^2 $dmol^{-1}$ at 222 nm, obtained with *Lunatia* and *Littorina* hemocyanins at pH 5.7 and 7.6, suggest fairly similar, low helical conformation with approximately 15–18% α-helical folding. Analysis of the wavelength-dependence data based on the reference parameters of Chen et al. (1972, 1974) suggests the further presence of about 50–60% β structure and 22–32% unordered

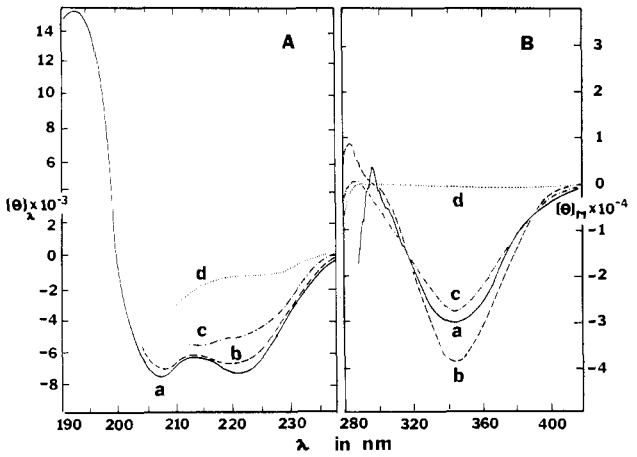

FIGURE 10: Circular dichroism spectra of *Littorina littorea* hemocyanin in the peptide-absorbing (A) and the copper-absorbing (B) spectral regions: (curves a) pH 5.7; (curves b) pH 10; (curves c) 8.0 M urea, pH 8.2; (curves d) 6.0 M GdmCl, pH 5.7.

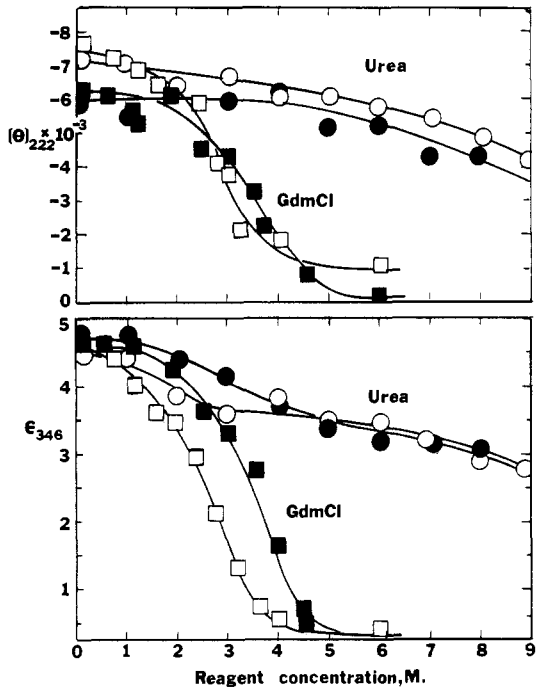

FIGURE 11: Effects of urea and GdmCl on the mean residue ellipticity at 222 nm ($[\theta]_{222}$) and the copper absorbance at 346 nm (ϵ_{346}) of *Littorina littorea* hemocyanin (open symbols) and *Lunatia heros* hemocyanin (filled symbols) investigated at pH 8.0 and 7.6, respectively.

chain folding in these two hemocyanins.

The molluscan hemocyanins are found to be very stable and resistant to the unfolding effects of urea and GdmCl. This is suggested by both our earlier findings with *Helix pomatia* α-hemocyanin, *Busycon canaliculatum* hemocyanin (Herskovits et al., 1983, 1985), and our present data of Figure 11. Neither urea nor high pH seems to alter very significantly the CD and copper absorbance spectra of *Littorina* and *Lunatia* hemocyanins despite the complete dissociation of the subunits, characterized by the molecular masses of about (4.4–4.8) × 10^5 daltons (Table II). Unfolding of the dissociated subunits is attained at GdmCl concentrations above 3.2 M (Figure 11). Both the CD and the absorbance data suggest little or no denaturation below 2 M concentrations, where most salt and GdmCl dissociation is observed (Figure 4). The data of Figures 5 and 6 indicate that the dissociation produced by urea occurs below 2–4 M concentrations. Significant changes in the CD data are seen only above these concentrations. This means that the observed dissociations with most of the reagents of this study are not complicated by the denaturation or unfolding of the hemocyanin subunits. The somewhat greater change in absorbance seen at 346 nm with urea (Figure 11) can be attributed to the slight lowering of the copper absorbance due to the decrease in turbidity of the dissociated hemocyanins (Heirwegh et al., 1961; Herskovits & Russell, 1984).

Discussion

The subunits organization of the hemocyanins of the two species of marine snails examined in this study is largely unaltered by NaCl and NaBr (Table I and Figure 4). In contrast to the behavior of α-hemocyanin of the land snail *Helix pomatia*, which is strongly dissociated by these salts (Engelborghs & Lontie, 1973; Herskovits & Russell, 1984), this behavior signifies the presence of the more salt-resistant β-type proteins in the hemolymph of these snails. On the basis of these results and the earlier finding of Wood (1973), who studied the ultracentrifugation behavior of three hemocyanins from the family Buccinidea, *Buccinum undatum*, *Neptunea antiqua*, and *Colus gracilis*, it would appear that the marine snails possess predominantly β-type hemocyanin chains. It should be noted, however, that some dissociation has been observed with *Lunatia heros* hemocyanin (Figure 4) in more concentrated NaBr solutions and that complete dissociation to dimeric and monomeric chains has been attained with the higher members of the Hofmeister series, NaI, $NaClO_4$, and GdmCl. As with the α-hemocyanin of *H. pomatia*, the order of effectiveness of these salts as dissociating reagents turns out to be the same. The lower resistance of the α-type chain assemblies to salt dissociation seems to represent a qualitative rather than a quantitive difference between subunit interactions of the two types of proteins. This can be attributed to the less hydrophobic character of the contact areas between the half-molecules or decamers.

Analysis of the salt dissociation data for *H. pomatia* α-hemocyanin suggested approximately 60–150 amino acid groups at the contact areas of each half-molecule, with predominantly polar interaction governing the up and down contacts in the whole molecule (Herskovits & Russell, 1984). However, with the much less dissociable β-type hemocyanin of *Littorina littorea* our estimates of hydrophobic groups using the ureas as dissociating probes gave only about 8–20 groups for each half-molecule, suggesting some, but comparatively modest, hydrophobicity for these contact areas of the hemocyanin of this species. As shown by the urea dissociation data of Figure 8, a much higher estimate of apparent groups (N_{app}) binding or interacting with urea could not be used to give a satisfactory account for the initial decline of the observed molecular weight data.

The urea series reagents are found to be fairly effective dissociating agents for both *Lunatia heros* and *Littorina littorea* hemocyanin. On the basis of the data of Figures 5 and 6, their ranking or order of effectiveness follows the order expected for the dismemberment of a hydrophobically stabilized subunit system. The same order of increasing effectiveness was observed with the dissociation of the half-molecules of *H. pomatia* α-hemocyanin, investigated in the presence of 0.5 M NaCl (Herskovits & Russell, 1984), and *Littorina littorea* hemocyanin, in the presence of 1.2 M urea (Figure 6B). The sedimentation data of Figure 2B and the molecular weight data at the low protein concentrations used for this study suggest that the whole hemocyanin particles are nearly 80% dissociated in 1.2 M urea and even more so at the al-

kylurea concentrations approaching the molecular weight transitions. With the relatively large N_{app} estimates of about 30–60 at the contact areas of the dimeric subunits forming the half-molecules (Herskovits & Russell, 1984; see also Figure 8), this suggests predominately hydrophobic stabilization of the side to side contacts in these cylindrically arranged particles. The nonhydrophobic or weakly hydrophobic characters of the up and down contacts between the half-molecules would require that the relatively sharp dissociation transitions seen with the urea series of reagents should also be attributed to the destabilization or weakening of the side to side contacts in the assembled whole molecules of hemocyanin. The dissociation effects of the higher members of the Hofmeister salts, NaI, $NaClO_4$, and GdmCl, could also be attributed to such destabilization of the hemocyanin structure, since these reagents are known to interact strongly with the peptide groups of protein chains (von Hippel & Schleich, 1969; Herskovits et al., 1977).

The absence of a biphasic character of the observed dissociation transitions seen with the two β-type hemocyanin of this study as well some other hemocyanins such as *Busycon canaliculatum* and *Urosalpinx cinerea* hemocyanins that we have managed to look at so far requires further comment. With the latter marine gastropod hemocyanins either we have different stabilization and dissociation patterns from the α-type hemocyanins, producing what amounts to two-state or two-species transitions with only whole molecules and one-tenth molecules present in solution and essentially no half-molecular intermediates present, or alternatively we have overlapping two-step transitions, with whole molecules dissociating to half-molecules and half-molecules producing one-tenth molecular weight fragments. Both the sedimentation and the light scattering data tend to support the latter hypothesis. The sedimentation data of Figure 2 show clearly more than two species present in the dissociation transition region produced by urea. Moreover, it is significant that in the simpler *Littorina* dissociation, where no one and a half molecules having sedimentation constants in the neighborhood of 130S appear to be present, the three species observed in the centrifuge appear to be of about the same proportion as those suggested by the species distribution plot obtained from the light-scattering data (Figure 9). In the presence of 1.5 M urea, for example, both techniques suggest the presence of about 20–35% half-molecules.[3] Such a large proportion of half-molecular weight intermediates are clearly inconsistent with a two-species scheme of hemocyanin dissociation.

The model calculations based on eq 2–6 are also very informative regarding the factors that govern the shape of the observed molecular weight transitions and the distribution of species that generates these transitions. The curve fitting and the related data of Figures 7 and 8 show that while both dissociation schemes can be used to give a correct account of the observed molecular weight, only the latter two-step transition can lead to the correct prediction of the distribution of species, which is also consistent with the ultracentrifugation data. Part of the reason for the observed merging or overlapping transitions is our finding that the apparent free energies of contact per half-molecule and per dimeric fragment, based on the observed $K_{w,app}$ values, are not very different, ranging from 5 to 6 kcal/mol for the former and 10 to 13 kcal/mol for the latter fragments. The apparent number of groups, N_{app}, is also instrumental in defining the character of the transition. Thus, the calculated curves of Figure 8, represented by the broken lines, show that as the number of interacting groups with the probe molecule at the less stable contact area of the protein assembly increase (from $N_{app}^{20,10}$ = 5–60 in Figure 8), the steepness in the initial decline in the molecular weights also increases. The biphasic character of the transition becomes clearly discernible as the number of groups characterizing the first transition approaches that of the second transition, in this case representing the side to side contacts within the half-molecules of hemocyanins. To show what influence a greater number of interacting amino acid groups will have on the observed dissociation transitions of molluscan hemocyanins, we have also included in this figure the dissociation curve of *H. pomatia* α-hemocyanin, obtained with sodium acetate as dissociating reagent (Herskovits & Russell, 1984). The contact energies calculated for this hemocyanin on the basis of the dissociation constants obtained with various salts (Herskovits & Russell, 1984) are not very different from the values obtained with *Littorina* hemocyanin. The actual values range from 6.1 to 6.3 kcal/mol and about 10–12 kcal/mol per half-molecule and dimeric fragment, respectively. Thus, the biphasic transitions seen with the salts clearly are not due to gross differences in stabilization of the two different sets of contact areas of these hemocyanins. It is important to note, however, that all our derived parameters are only apparent quantities subject to question unless independently verified, as in the case of the three-, rather than two-, species distribution of *Littorina* hemocyanin observed in the urea dissociation transition region. This is due to the problem of subunit heterogeneity encountered with both arthropod and molluscan hemocyanins (Di Giamberardino, 1967; Konings et al., 1969; Engelborghs & Lontie, 1973) and their complicating influence on both protein equilibria and reassociation or reversibility discussed in several recent papers (Van Holde et al., 1977; Herskovits et al., 1984; Siezen et al., 1984; Tai & Kegeles, 1984). Consequently, it needs to be emphasized that undue weight should not be given to our actual estimates of amino acid groups (N_{app}) at the contact areas of the subunits on the basis of our present analysis of the light-scattering data.

[3] On the basis of the fit of the light-scattering data, the uncertainties in these estimates are of the order of 10–15%.

References

Bonaventura, J., Bonaventure, C., & Sullivan, B. (1977) in *Oxygen and Physiological Function* (Jobsis, F., Ed.) Professional Information Library, Dallas, TX.

Brohult, S. (1947) *J. Phys. Colloid Chem. 51*, 206–217.

Brouwer, M., Ryan, M., Bonaventura, J., & Bonaventura, C. (1978) *Biochemistry 17*, 2810–2815.

Chen, Y. H., Yang, Y. T., & Martinez, H. M. (1972) *Biochemistry 11*, 4120–4131.

Chen, Y. H., Yang, Y. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.

Condie, R. M., & Langer, R. B. (1964) *Science (Washington, D.C.) 144*, 1138–1140.

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

Dijk, J., Brouwer, M., Coert, A., & Gruber, M. (1970) *Biochim. Biophys. Acta 221*, 467–479.

Doty, P., & Edsall, J. T. (1951) *Adv. Protein Chem. 6*, 35–121.

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

Ellerton, H. D., Ellerton, N. F., & Robinson, H. A. (1983) *Prog. Biophys. Mol. Biol. 41*, 143–248.

Engelborghs, Y., & Lontie, R. (1973) *J. Mol. Biol. 77*, 577–587.

Ghiretti-Magaldi, A., Salvato, B., Tallandini, L., & Beltramini, M. (1979) *Comp. Biochem. Physiol. A 62A*, 579–584.

Gielens, C., Preaux, G., & Lontie, R. (1975) *Eur. J. Biochem. 60*, 271-280.
Handbook of Chemistry and Physics (1979) 60th ed., CRC Press, Cleveland, OH.
Harrington, J. P., Pandolfelli, E. R., & Herskovits, T. T. (1973) *Biochim. Biophys. Acta 328*, 61-73.
Heirwegh, K., Borginon, H., & Lontie, R. (1961) *Biochim. Biophys. Acta 48*, 517-526.
Herskovits, T. T., & Russell, M. W. (1984) *Biochemistry 23*, 2812-2819.
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., & Herp, A. (1981a) *Biochim. Biophys. Acta 667*, 44-58.
Herskovits, T. T., San George, R. C., & Erhunmwunsee, L. J. (1981b) *Biochemistry 20*, 2580-2587.
Herskovits, T. T., Russell, M. W., & Carberry, S. E. (1984) *Biochemistry 23*, 1873-1881.
Herskovits, T. T., Carberry, S. E., & Villanueva, G. B. (1985) *Biochim. Biophys. Acta* (in press).
Konings, W. N., Siezen, R. J., & Gruber, M. (1969) *Biochim. Biophys. Acta 194*, 376-385.
Senozan, N. M., Landrum, J., Bonaventura, J., & Bonaventura, C. (1981) in *Invertebrate Oxygen Binding Proteins, Structure, Active Site and Function* (Lamy, J., & Lamy, J., Eds.) pp 703-717, Marcel Dekker, New York.
Siezen, R. J., Van Bruggen, E. F. J., Tai, M.-S., Crossin, M. C., & Kegeles, G. (1984) *Biophys. Chem. 19*, 99-112.
Svedberg, T., & Pedersen, K. O. (1940) *The Ultracentrifuge*, Oxford University Press, Oxford, England.
Tai, M.-S., & Kegeles, G. (1984) *Biophys. Chem. 19*, 113-120.
Van Bruggen, E. F. G., Wiebenga, E. H., & Gruber, M. (1962) *J. Mol. Biol. 4*, 1-7.
Van Holde, K. E., & Cohen, L. B. (1964) *Biochemistry 3*, 1802-1808.
Van Holde, K. E., & Miller, K. (1982) *Q. Rev. Biophys. 15*, 1-129.
Van Holde, K. E., Blair, D., Eldren, N., & Arisaka, F. (1977) in *Structure and Function of Hemocyanin* (Bannister, J. V., Ed.) pp 22-30, Spring-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 417-574, Marcel Dekker, New York.
Wood, E. J. (1973) *Biochim. Biophys. Acta 328*, 101-106.
Wood, E. J., Bannister, W. H., Oliver, C. J., Lontie, R., & Witters, R. (1971) *Comp. Biochem. Physiol., B: Comp. Biochem. 40B*, 19-24.

Structural Differences in the Two Calcium Binding Sites of the Porcine Intestinal Calcium Binding Protein: A Multinuclear NMR Study†

Hans J. Vogel,* Torbjörn Drakenberg, and Sture Forsén
Department of Physical Chemistry 2, University of Lund, Lund 220-07, Sweden
Joe D. J. O'Neil and Theo Hofmann*
Department of Biochemistry, University of Toronto, Toronto, Canada M5S 1A8
Received September 11, 1984; Revised Manuscript Received February 7, 1985

ABSTRACT: Cadmium-113 and calcium-43 NMR spectra of Cd^{2+} and Ca^{2+} bound to the porcine intestinal calcium binding protein (ICaBP; M_r 9000) contain two resonances. The first resonance is characterized by NMR parameters resembling those found for these cations bound to proteins containing the typical helix–loop–helix calcium binding domains of parvalbumin, calmodulin, and troponin C, which are defined as EF-hands by Kretsinger [Kretsinger, R. H. (1976) *Annu. Rev. Biochem. 45*, 239]. The second resonance in both spectra has a unique chemical shift and is consequently assigned to the metal ion bound in the N-terminal site of ICaBP. This site is characterized by an insertion of a proline in the loop of the helix–loop–helix domain and will be called the pseudo-EF-hand site. The binding of Cd^{2+} to the apo form of ICaBP is sequential. The EF-hand site is filled first. Both binding sites have similar, but not identical, affinities for Ca^{2+}: at a Ca^{2+} to protein ratio of 1:1, 65% of the ion is bound in the EF-hand site and 35% in the pseudo-EF-hand site. The two sites do not appear to act independently; thus, replacement of Ca^{2+} or Cd^{2+} by La^{3+} in the EF-hand site causes changes in the environment of the ions in the pseudo-EF-hand site. In addition, the chemical shift of Cd^{2+} bound to the EF-hand site is dependent on the presence or absence of Ca^{2+} or Cd^{2+} in the pseudo-EF-hand site. These data show the existence of interactions between the two calcium binding sites that may be of a cooperative nature. ^1H NMR studies demonstrate that the changes in the tertiary structure of ICaBP induced by saturating levels of Ca^{2+} and Cd^{2+}, respectively, are virtually indistinguishable. ^1H NMR studies also confirm the different modes of binding of the two cations.

The levels of the intracellular messenger Ca^{2+} undergo reversible changes that depend on hormonal or nerve impulses. A unique class of calcium binding proteins translates transient Ca^{2+} increases into metabolic or mechanical responses. For example, Ca^{2+}–calmodulin alters the efficiency of various metabolic pathways (Klee & Vanaman, 1982), Ca^{2+}–troponin C regulates contraction in skeletal and cardiac muscle (McCubbin & Kay, 1980), and parvalbumin appears to aid

† This work was supported by grants from the Medical Research Council of Canada and the Swedish Natural Science Research Council (NFR). H.J.V. is the recipient of a scholarship from the Swedish Natural Science Research Council.

0006-2960/85/0424-3870$01.50/0