Solution Studies on Heme Proteins: Subunit Structure, Dissociation, and Unfolding of *Lumbricus terrestris* Hemoglobin by the Ureas†

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ABSTRACT: The subunit structure, dissociation, and unfolding of the hemoglobin of the earthworm, *Lumbricus terrestris*, were investigated by light scattering molecular weight methods and changes in optical rotatory dispersion (at 233 nm) and absorption in the Soret region. Urea and the alkylureas, methyl-, ethyl-, propyl-, and butylurea, were employed as the reagents to cause both dissociation and unfolding of the protein. Analysis of the light scattering data suggests that the dissociation patterns as a function of hemoglobin concentration in the various dissociating solvents can be described in quantitative terms, either as an equilibrium mixture consisting of parent duodecamers and hexamers of 3×10^6 and 1.5×10^6 molecular weight (in 1–3 *M* urea, 1–2 *M* methyl- and ethylurea, and 1 *M* propylurea), as a mixture of hexamers and monomers, the latter with a molecular weight of 250000 (i.e., in 4 *M* urea), or as a mixture of all three species of duodecamers, hexamers, and monomers, seen in 2 *M* propylurea. Parallel studies by optical rotation and absorption measurements indicate that there is little or no unfolding of the subunits at urea and alkylurea concentrations where complete dissociation to hexamers and extensive dissociation to monomers can be achieved. Further splitting of the monomers (A subunits) to smaller fragments of one-third to one-quarter of the molecular weight of the monomers (B subunits) is seen in the presence of 7 and 8 *M* urea (pH 7) and in alkaline urea to propylurea solutions. Analysis of the dissociation data of duodecamers to monomers, based on equations used in studies of the urea and amide dissociation of human hemoglobin A from our laboratory, suggests few urea and alkylurea binding sites at the areas of hexamer contacts in the associated duodecameric form of *L. terrestris* hemoglobin. This suggests that hydrophobic interactions are not the dominant forces that govern the state of association of *L. terrestris* hemoglobin relative to polar and ionic interactions. The unfolding effects of the ureas, at concentrations above the dissociation transitions, are closely similar to their effects on other globular proteins, suggesting that hydrophobic interactions play an important role in the maintenance of the folded conformation of the subunits. Use of the Peller–Flory equation, with binding constants based on free energy transfer data of hydrophobic amino acid side chains and denaturation data used in previous denaturation studies, gave a relatively good account of the observed denaturation midpoints obtained with the various ureas supporting these conclusions.

Recent investigations from our and other laboratories have shown that the hydrophobic solutes such as the alkylureas (Herskovits et al., 1970a–c; Elbaum and Herskovits, 1974; Elbaum et al., 1974a,b; Bhat and Herskovits, 1975) and electrolytes of the Hofmeister series (see for example Von Hippel and Schleich, 1969; Kawahara et al., 1965; Guidotti, 1967; Hsu and Neet, 1973; McColl et al., 1971; Norèn et al., 1974) can destabilize the native conformation and subunit organization of proteins and protein assemblies, and thereby cause their unfolding and dissociation into subunits. Our studies of the denaturation and dissociation of the hemoglobins and hemoglobin aggregates found in sickle cells, by the ureas and amides, was prompted by the hope of being able to account for their effects in terms of the number and type of amino acids that are involved in the stabilization of the conformation and subunit organization of the hemoglobins in aqueous solution (Elbaum and Herskovits, 1974; Elbaum et al., 1974a,b; Bhat and Herskovits, 1975).

The large multisubunit hemoglobin of the common earthworm, *Lumbricus terrestris*, the subject of this and the accompanying study (Harrington and Herskovits, 1975), was investigated with this aim in mind. The native protein has a molecular weight of about 3 million (Svedberg and Eriksson, 1933; Roche et al., 1960; Levine, 1963; Rossi-Fanelli et al., 1970; Wiechelman and Parkhurst, 1972; Harrington et al., 1973) and it consists of 12 large subunits arranged in two hexagonal close-ring patterns placed on top of one another, with mean dimensions of 260 × 160 Å (Levine, 1963; Roche et al., 1960). Light scattering and other investigations including our earlier characterization and molecular weight measurements have shown that the intact hemoglobin can be readily dissociated into fragments of about one-twelfth of its initial mass (Levine, 1963; Rossi-Fanelli et al., 1970; Chiancone et al., 1972; Wiechelman and Parkhurst, 1972; Harrington et al., 1973). The effects of the alkylureas on the conformation and the state of association of the subunits of this hemoglobin which were examined by optical rotatory dispersion (ORD), absorbance, and light scattering measurements are reported in this paper, while the effects of the Hofmeister series of salts and guanidinium hydrochloride, investigated by the same methods, are presented in the accompanying paper (Harrington and Herskovits, 1975).

Materials and Methods

L. terrestris hemoglobin was isolated from the blood of earthworms according to the method of Rossi-Fanelli and coworkers (1970). Live 5–7-in. earthworms used were pur-

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chased from Ward's Natural Science Co., Rochester, N.Y. The final ultracentrifugation steps were carried out by centrifuging at 200000*g* for 2–3 hr at 0°. The red pellets of hemoglobin were redissolved in a minimum amount of 0.02 *M* potassium phosphate buffer (pH 7.0) containing 0.1 *M* KCl. The hemoglobin was either used immediately or stored frozen. No obvious changes in its properties were found for these latter preparations over a 6–8-week period as determined by spectroscopic and light scattering measurements.

Hemoglobin concentrations were determined spectrophotometrically on a Cary 14 recording spectrophotometer, using the percent extinction coefficient of 5.95 at 540 nm for the oxy form of the protein that corresponds to a molar value of 13.7×10^3 per mole of heme (Rossi-Fanelli et al., 1970). All the ureas and salts were of the purest commercial quality. With the exception of urea which was used without additional purification (Schwarz/Mann Ultra Pure grade), all the ureas were recrystallized from hot ethanol and dried in a vacuum oven. The water used was passed through an ion-exchange column and distilled directly from an all glass Corning still.

Light scattering measurements were made in a light scattering photometer of Brice's design (Brice et al., 1950) manufactured by Wood Mfg. Co., Newton, Pa., modified by the manufacturer for measurements at 630 nm in the red absorption region for hemoglobin studies (Elbaum and Herskovits, 1974). All the measurements were made on dialyzed solutions at constant chemical potential (Casassa and Eisenberg, 1964) clarified by filtration through 0.20-μ glass fiber filters as previously described (Harrington et al., 1973; Elbaum et al., 1974). Urea solutions were prepared by volumetric dilution using concentrated solutions of diluent and protein both containing 0.1 *M* KCl and 0.02 *M* phosphate buffer (pH 7.0) followed in most cases by overnight dialysis in the cold. It has been shown previously that dialysis equilibrium was usually reached after 2–3 hr (Elbaum and Herskovits, 1974). Most of the solutions were dialyzed separately.

Light scattering measurements were made in the concentration ranges, c, of 0.1–1.7 g/l. at an angle θ of 90° relative to the incident beam. For particles having mean dimensions less than 10% of the wavelength of the scattering light, the light scattering data can be analyzed by means of the familiar relationship (Doty and Edsall, 1951)

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

where R_θ is the Rayleigh's ratio related to the measured turbidity of the protein solution in excess of solvent or dialysate, M_w is the weight average molecular weight, and K' is the light scattering constant which also incorporates the Cabannes depolarization correction, $K' = 2\pi^2 n_0^2 (\partial n/\partial c)_\mu^2 / N_A \lambda^4 [(6 + 6\rho)/(6 - 7\rho)]$, where n_0 is the refractive index of the solvent, $(\partial n/\partial c)_\mu$ is the refractive index increment at constant chemical potential obtained on dialyzed solutions, N_A is Avogadro's number, and λ is the wavelength of the scattering light employed for the measurements.

The $(\partial n/\partial c)_\mu$ and the n_0 values of the various urea and salt solutions at 630 nm were determined directly in the light scattering photometer equipped with a Wood differential refractometer attachment. The refractometer was calibrated using the refractive index data of Kruis as previously described (Harrington et al., 1973). Table I lists the $(\partial n/\partial c)_\mu$, n_0, ρ (the depolarization ratio), as well as other physical data obtained on *L. terrestris* oxyhemoglobin.

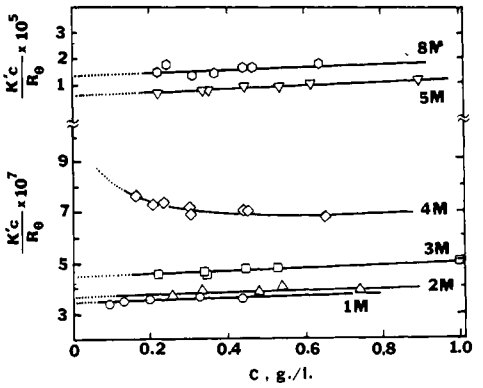

FIGURE 1: Light scattering plots of *L. terrestris* oxyhemoglobin in the presence of 0–8 *M* urea, 0.1 *M* KCl, and 0.02 *M* phosphate (pH 7.0) buffer, 25 ± 2°.

The optical rotatory dispersion (ORD) measurements were made on a Cary 60 recording spectropolarimeter as previously described (Harrington et al., 1973; Herskovits et al. 1970a).

Results

Light Scattering. The effects of increasing urea concentration on the scattering behavior of the duodecameric structure of *L. terrestris* oxyhemoglobin at pH 7.0 are shown in Figure 1. According to eq 1 the decrease in observed scattering with increasing dissociation of the protein into subunits will result in increased displacement to higher $K'c/R_\theta$ values of the set of related $K'c/R_\theta$ vs. c curves reflecting the decrease in molecular weight with increasing urea concentration. The changes in the calculated molecular weights based on the data of this figure, summarized in Table I, suggest an initial splitting of the parent protein to half-molecules consisting of six subunits, first seen by Levine (1963) in the electron microscope, followed by further dissociation to one-twelfth and lower molecular weight units above 4 *M* urea. The observed changes in the mean residue rotation, $[m']_{233}$, and the absorbance in the Soret region at 417 nm, also shown in Table I, suggest that some unfolding of the dissociated subunits may also have occurred at higher urea concentrations. The minimum molecular weight of 77000 obtained in 8 *M* urea represents the further splitting of the individual subunits into smaller fragments of one-third to one-fourth of their initial molecular weight of 250000.

As suggested by the data of Figure 2 and Table I, methyl-, ethyl-, and propylurea also cause the splitting and dissociation of *L. terrestris* hemoglobin, as predicted on the basis of eq 14 of the Discussion.

Model Calculations. Depending on the urea or alkylurea concentration and the pH of the hemoglobin solutions, the light scattering data can be described in quantitative terms either as a mixture of duodecamers and hexamers (up to 3 *M* urea, 1 *M* propylurea, and 1–2 *M* methyl- and ethylurea), hexamers and monomers (in 4 *M* urea for example), or as a mixture of all three species of duodecamers, hexamers and monomers, seen in 2 *M* propylurea. The duodecamer–hexamer equilibrium species present can adequately describe the observed light scattering behavior shown in Figure 3A. In general, in terms of α, the degree of dissociation of a subunit protein of molecular weight M_1, dissociating into m subunits is given by the expression

$$\alpha = (m/(m - 1))(1 - M_w/M_1) \qquad (2)$$

Table I: Light Scattering, ORD, and Absorbance Data of *Lumbricus terrestris* Oxyhemoglobin in Various Ureas at Neutral pH 7.0 and at pH 10–10.3.

Solvent	Refractive Index Increment $(\partial n/\partial c)_\mu$	Refractive Index at 630 nm, n_{630}	Depolarization, Ratio, ρ	Cabannes Factor $(6+6\rho)/(6-7\rho)$	Mol Wt, M_w	$[m']_{233}$	$\epsilon_M \times 10^{-5}$ at 417 nm
Neutral pH, 7.0							
Water, 0.1 *M* KCl, 0.02 *M* phosphate	0.193 ± 0.003	1.327	0.00865	1.020	2.92 ± 0.1 × 10^6	−5430	1.13
1 *M* urea	0.191	1.337	0.00792	1.017	2.96 × 10^6	−5680	1.15
2 *M* urea	0.189	1.345	0.00857	1.019	2.86 × 10^6	−5800	1.15
3 *M* urea	0.188	1.354	0.00702	1.015	2.27 × 10^6	−5625	1.14
4 *M* urea	0.185	1.366	0.01160	1.025	1.14 × 10^6 [a]	−5200	1.12
5 *M* urea	0.182	1.373	0.05460	1.126	0.22 × 10^6	−5045	0.86
8 *M* urea	0.167	1.397	0.11490	1.287	0.77 × 10^5	−2560	0.47
1 *M* methylurea	0.190	1.339	0.00927	1.020	2.90 × 10^6	−5750	1.13
2 *M* methylurea	0.183	1.349	0.00754	1.017	2.51 × 10^6	−5800	1.15
1 *M* ethylurea	0.190	1.342	0.00817	1.018	2.90 × 10^6	−5500	1.14
2 *M* ethylurea	0.179	1.352	0.00975	1.021	2.56 × 10^6	−5690	1.13
1 *M* propylurea	0.190	1.344	0.00820	1.018	2.94 × 10^6	−5595	1.13
2 *M* propylurea	0.177	1.356	0.01214	1.027	2.00 × 10^6 [a]	−5160	1.11
0.2 *M* butylurea	0.192	1.332	0.00940	1.020	2.96 × 10^6	−5600	1.15
Alkaline pH, 10.1–10.3 [b]							
0.05 *M* borate, 0.1 *M* KCl (pH 10.1)	0.190	1.327	0.04190	1.095	2.80 × 10^5	−5200	1.08
1 *M* urea (pH 10.1)	0.153	1.337	0.04190	1.095	1.82 × 10^5	−5200	1.07
1 *M* methylurea, (pH 10.1)	0.154	1.339	0.04190	1.095	1.79 × 10^5	−4625	0.89
1 *M* ethylurea (pH 10.2)	0.153	1.341	0.04190	1.095	1.79 × 10^5	−4470	0.74
1 *M* propylurea (pH 10.3)	0.150	1.344	0.04190	1.095	1.82 × 10^5	−4070	0.70

[a] Due to curvature in the $K'c/R_\theta$ vs. c results of 4 *M* urea and 2 *M* propylurea data (see Figures 1 and 2) the M_w given for these two solvents represent values at a single concentration, c = 0.8 g/l. The rest of the data given represent values extrapolated to $c \rightarrow 0$. [b] Light scattering measured at 10 ± 2°. The rest of the results are based on 25 ± 2° measurements.

With this definition of α the light scattering eq 1 can be expressed as (Elbaum and Herskovits, 1974):

$$\frac{K'c}{R_\theta} = \frac{1}{M_1[1-\alpha(m-1)/m]} + 2B'c \qquad (3)$$

and the experimental data can be fitted using the dissociation constant, K_{Diss}, to define α, where

$$K_{Diss} = \frac{m^m \alpha^m c^{m-1}}{(1-\alpha)(M_1)^{m-1}} \qquad (4)$$

For the dissociation of duodecamers to hexamers and duodecamers to monomers, M_1 has been taken as 3 × 10^6, while m is equal to 2 and 12, respectively. For the dissociation of hexamers to monomers M_1 is 1.5 × 10^6 and m is equal to 6. Figure 3C presents an example of the latter fit of the data obtained with 4 *M* urea as the dissociating agent, using a dissociation constant, $K_{6,1} = 2 \times 10^{-36}$.

A more complex situation is encountered in the case of the dissociation of *L. terrestris* hemoglobin by 2 *M* propylurea, shown in Figure 3B, suggesting the presence of all three species, duodecamers, hexamers, and monomers. The dissociation reaction and the species concentrations present can be expressed as

$$Hb_{12} \overset{K_1}{\rightleftharpoons} 2Hb_6 \overset{K_2}{\rightleftharpoons} 12Hb_1 \qquad (5)$$

$$c(1-\alpha_1) \quad c\alpha_1(1-\alpha_2) \quad c(\alpha_1\alpha_2)$$

where α_1 is the weight fraction of duodecamers dissociating to hexamers and monomers and α_2 is the fraction of monomers formed. The two equilibrium constants describing reaction 5 can then be written as

$$K_1 = K_{12,6} = \frac{4c[\alpha_1(1-\alpha_2)]^2}{(1-\alpha_1)M_{12}} \qquad (6)$$

and

$$(K_2)^{1/2} = K_{6,1} = \frac{4.67 \times 10^4 c^5 (\alpha_1\alpha_2)^6}{[\alpha_1(1-\alpha_2)](M_6)^5} \qquad (7)$$

In order to calculate the α_1 and α_2 values as a function of concentration, c, required for fitting the light scattering data, we found it convenient to express the pairs of related constants, $K_{12,6}(M_{12})/4c = [\alpha_1(1-\alpha_2)]^2/(1-\alpha_1)$ and $K_{6,1}(M_6)^5/(4.67 \times 10^4 c^5) = (\alpha_1\alpha_2)^6/[\alpha_1(1-\alpha_2)]$ and estimate α_1 and α_2 by successive approximations until the best fit values are obtained, dictated by the choice of the equilibrium constants, $K_{12,6}$ and $K_{6,1}$. Based on the definition of the weight average molecular weight, M_w, with

$$M_w = [(c_{12}M_{12} + c_6M_6 + c_1M_1)/c] = [(1-\alpha_1)M_{12} + \alpha_1(1-\alpha_2)M_6 + \alpha_1\alpha_2M_1] \qquad (8)$$

the light scattering eq 1 can be expressed in terms of these α_1 and α_2 values and used for the data fitting. The latter equation has the form

$$K'c/R_\theta = [(1-\alpha_1)M_{12} + \alpha_1(1-\alpha_2)M_6 + \alpha_1\alpha_2M_1]^{-1} + 2B'c \qquad (9)$$

where M_{12}, M_6, and M_1 represent the molecular weight of the species present taken as 3 × 10^6, 1.5 × 10^6, and 0.25 × 10^6, respectively. The second virial coefficient B' with a value of 5 × 10^{-9} l. mol/g^2 was found to give the best rep-

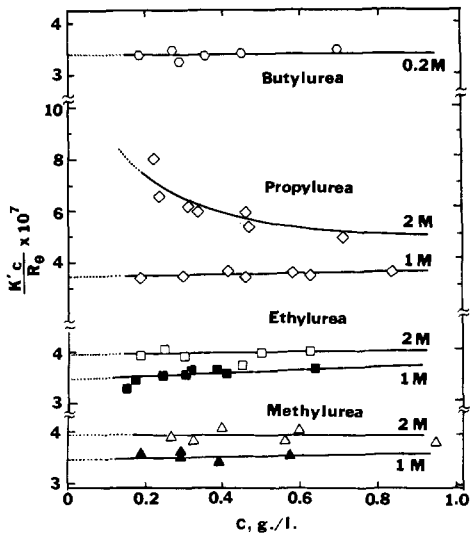

FIGURE 2: The effects of 0.2–2 *M* alkylureas on the light scattering of *L. terrestris* oxyhemoglobin. Solvent conditions the same as in Figure 1.

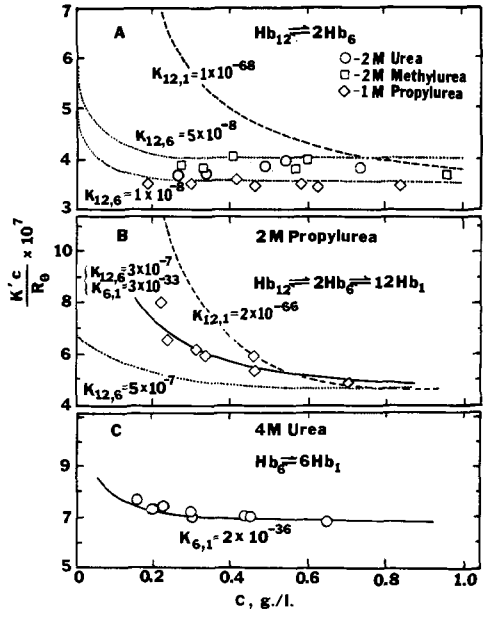

FIGURE 3: The effects of alkylurea dissociation on the light scattering data of *L. terrestris* oxyhemoglobin fitted according to eq 3 and 9. Solvent conditions the same as in Figure 1. (A) Duodecamer to hexamer dissociation of the 2 *M* urea, 2 *M* methylurea, and 1 *M* propylurea data with dissociation constants, $K_{12,6} = 1 \times 10^{-8}$ to 5×10^{-8}. For comparison the duodecamer to monomer scheme of dissociation a curve with $K_{12,1} = 1 \times 10^{-68}$ is also included in this part of the figure. (B) Duodecamer–hexamer–monomer dissociation in 2 *M* propylurea fitted with $K_{12,6} = 3 \times 10^{-7}$, $K_{6,1} = 3 \times 10^{-33}$ (eq 6 and 7). For comparison the duodecamer–monomer and hexamer–monomer schemes of dissociation with $K_{12,1} = 2 \times 10^{-66}$ and $K_{6,1} = 5 \times 10^{-7}$ curves are also included in this part of the figure. (C) Hexamer to monomer dissociation in 4 *M* urea fitted with $K_{6,1} = 2 \times 10^{-36}$.

Table II: A Summary of the Dissociation Constants and Free-Energy Change for the Subunit Dissociation of *Lumbricus terrestris* Oxyhemoglobin.

Dissociating Medium	$K_{12,6}$[a] (M^{-1})	$K_{6,1}$[a] (M^{-6})	$\Delta G^\circ_{6,1}$ (kcal/mol)
0.1 *M* KCl, 0.02 *M*, pH 7.0 phosphate	3×10^{-9} [b]		11.6
2 *M* urea	3×10^{-8}		10.3
3 *M* urea	8×10^{-8}		9.7
4 *M* urea		2×10^{-36}	
2 *M* methylurea	5×10^{-8}		10.0
1 *M* ethylurea	1×10^{-8}		10.9
2 *M* ethylurea	5×10^{-8}		10.0
1 *M* propylurea	1×10^{-8}		10.9
2 *M* propylurea	3×10^{-7}	3×10^{-33}	8.9

[a] Based on the best fit of the data, with examples shown in Figure 3 utilizing eq 2–9 of the text. [b] Extrapolated value based on Figure 7A.

resentation of the data shown in Figure 3. This value is five times higher than the hard sphere value of $4\bar{V}/M_{12} = 1 \times 10^{-9}$ l. mol/g^2. Table II presents a summary of our analysis of the light scattering data of Figures 1–3, including estimates of the free energy change, ΔG°, based on the $K_{12,6}$ values obtained. The distribution of species for two of the more interesting dissociating solvents, 2 *M* methylurea and 2 *M* propylurea, are shown as a function of hemoglobin concentration in Figure 4. The weight fractions, f_w, of duodecamer ($1 - \alpha_1$), hexamer ($\alpha_1(1 - \alpha_2)$), and monomer ($\alpha_1\alpha_2$) were calculated using the best fit α_1 and α_2 values generated by fitting the experimental data of 2 *M* methyl- and 2 *M* propylurea shown in Figure 3A and B. Equations 3, 4, 6, 7, and 9 were used for these calculations.

Denaturation Studies. Figure 5 presents a comparison of the denaturation profiles of *L. terrestris* hemoglobin obtained with the various ureas at pH 7.0. Both the ORD and the absorbance of the protein solutions in the Soret region were employed to follow the unfolding transition. The ORD data were used to calculate the apparent equilibrium constant of the unfolding reaction, $K_{App} = f_D/(1 - f_D)$, where f_D is the fraction of the denatured protein, determined by extrapolation of the base lines of the native and the unfolded forms of the protein into the denaturation region (Sturtevant and Tsong, 1969; Greene and Pace, 1974). The values of K_{App} were used to characterize the transitions based on the empirical eq 10, defining the apparent order of the transition, n, and also eq 11 of Aune and Tanford (1969):

$$K_{App} = C[D]^n \quad (10)$$

$$\Delta G^\circ_U = \Delta G^\circ_{U,w} - \Delta nRT \ln (1 + K_B[D]) \simeq \Delta G^\circ_{U,w} - \Delta nRTK_B[D] \quad (11)$$

In eq 10 and 11, C represents a constant characteristic of the protein–denaturant system, [D] is the concentration of the denaturant, ΔG°_U and $\Delta G^\circ_{U,w}$ are the free energies of unfolding in the presence and absence of denaturant, K_B is the binding constant of the denaturant to the average amino acid site, and Δn is the difference in the apparent number of binding sites in the unfolded and the native protein. Figure 6 shows plots of our data based on eq 10 and 11 with the derived n, Δn, and $\Delta G^\circ_{U,w}$ parameters based on these plots being summarized in Table III. The binding constants required for the Δn estimates are also listed in this table. Group additivity of the hydrophobic, $K_{H\Phi}$, and polar, K_P, contributions to the binding constant calculated according to the expression

$$K_B = K_{H\Phi} + K_P \quad (12)$$

was assumed, as described previously (Herskovits et al., 1970c; Elbaum et al., 1974). It is worth noting that the average n and Δn values of 6.5 ± 0.6 and 37 ± 4 obtained with this multichain hemoglobin are somewhat higher than the corresponding values of 5.0 ± 1 and 27 ± 2 obtained

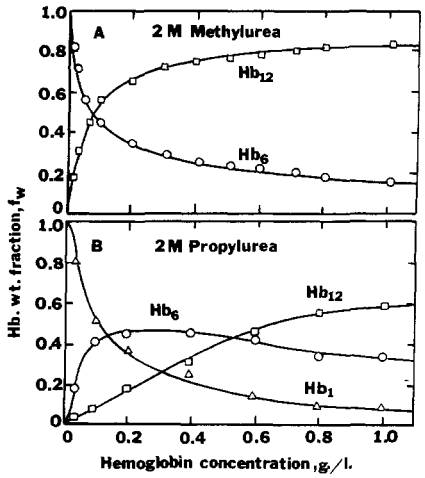

FIGURE 4: Species distribution of *L. terrestris* duodecamers (Hb_{12}) and hexamers (Hb_6) in 2 *M* methylurea solutions (A) and duodecamers, hexamers, and monomers (Hb_1) in 2 *M* propylurea solutions (B) based on the analysis of the light scattering data of Figure 3A and B by means of eq 3 and 9. The weight fraction of species are, $f_{12} = (1 - \alpha)$ or $(1 - \alpha_1)$, $f_6 = \alpha$ or $\alpha_1(1 - \alpha_2)$, and $f_1 = \alpha_1\alpha_2$, respectively.

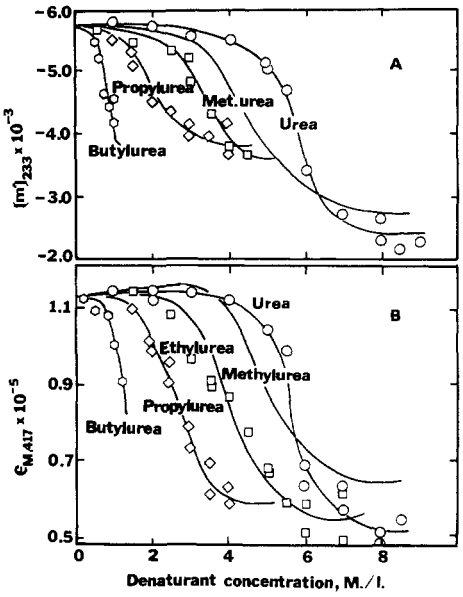

FIGURE 5: The denaturation of *L. terrestris* oxyhemoglobin by various ureas at neutral pH (7.0) followed by changes in the mean residue rotation, $[m']_{233}$ (ORD), and absorbance changes at the Soret 417-nm band. For reasons of crowding the methylurea data points are not given. Protein concentrations ranged from 0.011 to 0.024%. Solvent conditions and temperature (25°) are the same as in Figure 1.

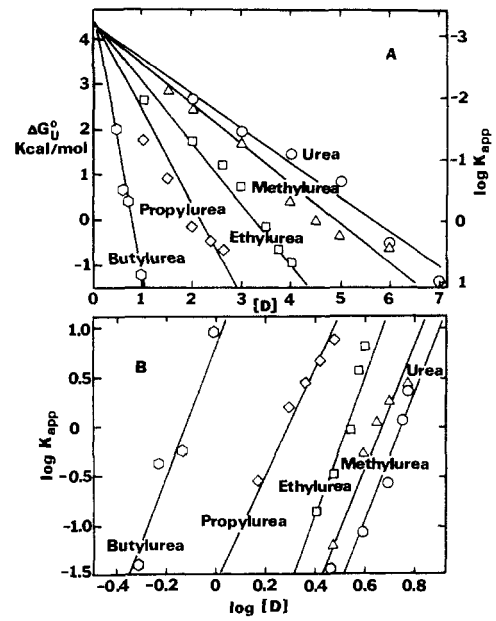

FIGURE 6: $\Delta G^\circ_{U,w}$ and log K_{App} vs. [D] plots (A) and log K_{App} vs. log [D] plots (B) of the *L. terrestris* hemoglobin denaturation data of Figure 5A. These plots are based on eq 10 and 11 of the text, with slopes proportional to n and Δn listed in Table III.

Table III: Denaturation Parameters of Neutral Solutions (pH 7.0) of *Lumbricus terrestris* Oxyhemoglobin Based on Eq. 10–13.

Denaturant	K_B [a]	S_m [b]	n [c]	Δn [c]	$\Delta G^\circ_{U,w}$ [c] (kcal/mol)
Urea	0.032	5.6	6.8	40	
Methylurea	0.042	4.6	6.4	35	
Ethylurea	0.061	3.7	7.2	37	
Propylurea	0.108	2.1	5.5	33	
Butylurea	0.232	0.85	6.4	42	
Av values			6.5 ± 0.6	37 ± 4	4.4 ± 0.3

[a] Parameters taken from Elbaum et al. (1974a). [b] Denaturation midpoints based on the averages of the ORD and Soret absorbance values of Figure 5. [c] n, Δn, and $\Delta G^\circ_{U,w}$ parameters based on the ORD data of Figure 6 plotted according to eq 10 and 11.

with the four-chain human oxyhemoglobin, and the values of 5.2 ± 0.3 and 27 ± 3 of the single chain *Glycera dibranchiata* oxyhemoglobin (E. R. Pandolfelli and T. T. Herskovits, unpublished results).

The relationship

$$S_m = (\Delta T_m \Delta h / R T_m T_m^0 \bar{\nu}) / K_B \qquad (13)$$

based on the theories of Peller (1959) and Flory (1957) related to the unfolding transition of biopolymers was also used to characterize the denaturation transitions of *L. terrestris* hemoglobin seen in Figure 5. In eq 13, T_m, T_m^0, ΔT_m, Δh, and $\bar{\nu}$ represent the midpoints of the denaturation transitions in the presence and in the absence of denaturant, the difference in T_m^0 and T_m, the enthalpy of unfolding per average amino acid, and the effective number or fraction of binding sites per amino acid, respectively. Table IV of the Discussion presents a comparison of the observed denaturation midpoints, based on the data of Figure 5, and the calculated S_m values using the binding constants given in Table III, that have also been employed in previous investigations from our laboratory (Herskovits and Harrington, 1972; Elbaum and Herskovits, 1974; Elbaum et al., 1974a; Herskovits and Bowen, 1974).

Discussion

Subunit Organization and Dissociation of L. terrestris Hemoglobin. The electron microscopy studies of Levine (1963) and Guerritore et al. (1965) on *L. terrestris* hemoglobin and other erythro- and chlorocruorins have shown that the common duodecamer structure can be altered by pH or aging, with resultant dissociation of the parent proteins to half-molecules (hexamers) and smaller fragments of one-twelfth of the initial molecular weight. Investigations from our laboratory have shown that the decrease in molecular weight from about 3×10^6 to 240000–250000 due to an increase in pH has essentially no effect on the folded

conformation of the fragments, as reflected by their optical rotation (ORD) and circular dichroism (CD) spectra (Harrington et al., 1973). For example, at pH 7.0 the ORD and CD extrema at 233 and 207–219 nm have mean values of −5430 and −15750 deg cm^2/dmol that are not significantly different from the pH 10.1 values of −5550 and −15750 deg cm^2/dmol, whereas the light scattering molecular weight has dropped sharply to 240000. The ureas are less effective dissociating agents than alkali or salts of the Hofmeister series described in the accompanying paper (Harrington and Herskovits, 1975). Their effects on the light scattering behavior of *L. terrestris* hemoglobin show clearly that (see Figures 3 and 4) the dissociation of the duodecameric parent structure of the protein in solution can be best described by the duodecamer–hexamer to monomer equilibrium scheme given by eq 5. The elegant kinetic studies of Goss et al. (1975) by light scattering stopped-flow methods are consistent with the same mechanism of product species produced by pH changes.

In concentrated urea solutions *L. terrestris* hemoglobin is both unfolded and dissociated into fragments that are smaller than the 250000 subunits (Table I). The minimum molecular weight of 77000 obtained in 8 *M* urea solutions is consistent with the electron microscopic observations of Guerritore et al. (1965) and the model proposed by Rossi-Fanelli et al. (1970) for the subunit organization of the erythrocruorins. This model views the large hemoglobin subunits A as consisting of three smaller structures B accommodating a total of six heme groups or two hemes per smaller B subunit. However, the gel filtration studies of Shlom and Vinogradov (1973) on *L. terrestris* hemoglobin suggest the presence of at least six different polypeptide chains of molecular weights of 12000–37000, and thus a much greater complexity of substructural organization of the 12 major subunits. The studies of Wiechelman and Parkhurst (1972) on *L. terrestris* hemoglobin and those of Waxman (1971) on *Arenicola crista* hemoglobin are more consistent with a four B-subunit organization. The higher molecular weight of 77000 seen in our experiments in the presence of 8 *M* urea could thus represent incomplete dissociation or the results of some aggregation.

Subunit Dissociation by the Ureas. Relative to their effects on subunit structure of human hemoglobin A, the ureas have only moderate effects on the duodecamer structure of *L. terrestris* hemoglobin. For example, at a hemoglobin concentration of 1 g/l. 1 *M* urea and 1 *M* propylurea cause approximately 30 and 92% dissociation of human CO-hemoglobin A to half-molecules of α,β subunits. The corresponding dissociation of *L. terrestris* oxyhemoglobin by these ureas is only marginal, with essentially no detectable changes in the light scattering molecular weights at the 1 molar level of dissociating agent. Moderate changes in molecular weights and dissociation are seen at higher levels of urea and alkylurea concentrations (Table I).

Since the effects of pH and salts are much more pronounced on the state of association of *L. terrestris* hemoglobin relative to the effects of the ureas (Harrington et al., 1973; Harrington and Herskovits, 1975), it is reasonable to assume that electrostatic and polar interactions dominate the stabilization energy of contact between the two hexameric halves of the parent protein. Hydrophobic interactions can thus be viewed as providing only a limited source of stabilization energy.

A more quantitative assessment of the role of hydrophobic interactions in terms of the average number of amino

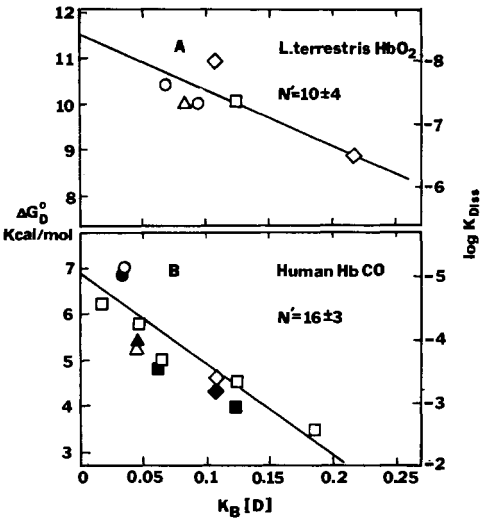

FIGURE 7: $\Delta G°_D$ and log K_{Diss} vs. K_B[D] plots of *L. terrestris* oxyhemoglobin (A) and human carboxyhemoglobin (B) based on eq 14 of the text. The dissociation constants of these two hemoglobins to half-molecules of hexamers or $\alpha\beta$ dimers are taken from Table II of this paper and Table VI of Elbaum and Herskovits (1974). Open symbols represent light scattering results while the closed symbols are osmometric results; these are: urea (○,●), methylurea (△,▲), ethylurea (□,■), and propylurea (◇,◆).

acid contacts that are disrupted as a result of dissociation by the ureas can be obtained by means of the relation

$$\Delta G°_D = \Delta G°_{D,w} - mN'RTK_B[D] \qquad (14)$$

employed in previous investigations from our laboratory of the urea and amide dissociation of human hemoglobin A (Elbaum and Herskovits, 1974; Bhat and Herskovits, 1975). In the above equation, $\Delta G°_D$ and $\Delta G°_{D,w}$ represent the standard free energy of dissociation of the parent protein in the presence and absence of dissociating agent [D], m is the number of subunits or fragments formed upon dissociation (i.e., $m = 2$ for the splitting of the *L. terrestris* duodecamer into hexamers) having N' independent and equivalent binding sites that are exposed as a result of dissociation, and K_B is the binding constant of the reagent for the average amino acid site exposed. According to this equation, the plot of $\Delta G°_D$ or log K_{Diss} vs. [D] or K_B[D] should be linear, having a slope that is proportional to N'. Figure 7 presents such plots for the dissociation of *L. terrestris* hemoglobin to hexamers by the ureas based on the K_{Diss} and K_B data taken from Tables II and III. For reasons of comparison we have also included in this figure the urea and alkylurea dissociation data obtained on human CO-hemoglobin (Elbaum and Herskovits, 1974) analyzed by use of the same equation. For *L. terrestris* hemoglobin the slope of the curve leads to an N' value of 10 ± 4 per hexamer formed. The corresponding N' value for human hemoglobin that also dissociates into half-molecules, but of much lower molecular weight (i.e., 32230) and presumably of much smaller area of contact, is 16 ± 3. The X-ray crystallographic studies of Perutz and coworkers (Perutz, 1965; Perutz et al., 1968) indicate that 19 amino acid contacts and probably two polar salt-bridge type contacts are disrupted as a result of the splitting of horse hemoglobin into half-molecules of $\alpha\beta$ dimers. Of the newly exposed groups 14 are hydrophobic or nonpolar and 5 are of ionic character. These groups are: 1 Trp, 2 Tyr, 1 Leu, 3 Val, 2 Pro, 2 Arg, 1 His, 2 Thr, and 1 Gln, 1 Asn, 1 Glu, and 2 Asp.

Table IV: A Comparison of the Experimental and Calculated Denaturation Midpoints of *Lumbricus terrestris* Oxyhemoglobin, Human Hemoglobin A, Horse Heart Cytochrome *c*, and Sperm Whale Myoglobin at 25°.

Denaturant	Human oxyhemoglobin[a] ORD	Cytochrome *c*[a] Soret Abs.	Myoglobin[a] Soret Abs.	*L. terrestris* Oxyhemoglobin, pH 7.0 ORD	Soret Abs.	Calcd Eq 13[b]	*L. terrestris* Oxyhemoglobin, pH 10.1 ORD	Soret Abs.	Calcd Eq 13[b]
Urea	6.5	6.6	6.6	5.8	5.7	6.1	2.6	2.5	1.9
Methylurea	4.5	5.6	5.6	4.8	4.7	4.6	1.3	1.5	1.4
Ethylurea	3.0	4.7	3.8	3.3	4.0	3.2	0.9	0.9	0.98
Propylurea	1.6	2.3	1.8	2.1	2.5	1.8	0.5	0.3	0.56
Butylurea	0.5	0.7	0.6	0.8	~0.9	0.8	0.25	0.25	0.26

[a] The hemoglobin data are from Elbaum et al. (1974a) while the cytochrome *c* and myoglobin data are from Herskovits et al. (1970b). [b] In the absence of Δh and T_m data the same ($\Delta T_m \Delta h / RT_m T_m^\circ$) value of 0.136 was used as in our previous investigations (Herskovits et al., 1970b; Elbaum et al., 1974a) with $\bar{\nu} = 0.70$ for the pH 7.0 calculations and ($\Delta T_m \Delta h / RT_m^\circ \bar{\nu}$) = 0.065 for the pH 10.1 calculations. K_B data of the same references were used that are given in column 1 of Table III.

With the splitting of *L. terrestris* hemoglobin parallel or perpendicular to the planes of the two hexagonal ring structures that are in contact with each other (Levine, 1963), the N' value of 10 ± 4 would only represent about one to three exposed binding sites per monomer of 250000 in each half-molecule. This suggests that the contact areas between the two hexamers contain few hydrophobic amino acid sites. Thus it is reasonable to assume that hydrophobic interactions contribute only secondarily to the energy of stabilization of the intact duodecamers.

Similar analysis of our light scattering data could not be made on the dissociation of hexamers to monomers observed at higher urea and alkylurea concentrations. The changes in the ORD and Soret absorbance parameters indicate that at neutral pH some denaturation occurs before much dissociation is seen to monomers (Table I). The studies of Goss et al. (1975) on *L. terrestris* methemoglobin suggest that this form appears to be hexameric. Also at pH 8.4 and 9.0 we have found that the molecular weights of the oxy form of *L. terrestris* hemoglobin are in the ranges of 2.4 to 1.0 × 10^6 (Harrington et al., 1973), not far from the expected value of 1.5 × 10^6, characteristic of hexamers. Further work will be required to give us information concerning the forces governing the intersubunit contact in the two hexamers.

Denaturation. Despite their ineffectiveness as subunit dissociating agents for *L. terrestris* hemoglobin, the ureas are found to be effective denaturing agents. The effectiveness of the urea series of solutes as unfolding or denaturing reagents is reflected by the location of the midpoints of the denaturation transitions, shown in Figure 5. The denaturation midpoints, S_m, obtained with the ureas on this multichain hemoglobin are comparable to the midpoints obtained with the four-chain human hemoglobin and the single chain *Glycera dibranchiata* hemoglobin, as well as myoglobin and other globular proteins investigated in our and other laboratories (Herskovits et al., 1970a–c; Elbaum et al., 1974a; Puett, 1973; Greene and Pace, 1974).

Previous investigations from our laboratory have established that in the case of globular proteins whose tertiary structure is stabilized by hydrophobic interactions, the unfolding or denaturation process will be enhanced as a result of the increasing hydrocarbon content of the unfolding reagent used (Herskovits et al., 1970a–c; Elbaum et al., 1974a). The close similarity of the effects of the alkylureas on the denaturation midpoints, S_m, of *L. terrestris* and human hemoglobin, myoglobin, and cytochrome *c* compared in Table IV suggests that hydrophobic interactions play an equally important role in the maintenance of the folded conformation of the subunits of this hemoglobin as in cases of other globular proteins investigated. The X-ray crystallographic structures of sperm whale myoglobin and horse heart cytochrome *c* (Kendrew, 1962; Dickerson et al., 1967; Dickerson and Geis, 1969) show that the interior regions of these two proteins are made up nearly entirely of nonpolar amino acid residues, with almost all the polar and ionic groups situated at the surface of the proteins. Dickerson and coworkers (1967) have cited cytochrome *c* as an example of the "hydrophobic drop" model for the internal folding of globular proteins.

The effects of a given urea on the midpoint of the denaturation transition, S_m, can be evaluated by means of eq 13 using the binding constants of our previous investigations (Herskovits et al., 1970c; Elbaum et al., 1974). According to this equation the denaturation midpoint should be inversely proportional to K_B. With progressively larger nonpolar contribution, $K_{H\Phi}$ to the binding constant of a given series, the higher more hydrophobic compounds, such as propyl and butylurea, should be the more effective denaturants. The relatively close agreement found between the experimental and calculated S_m values (Table IV) supports the above conclusion concerning the importance of hydrophobic interactions related to the folding of the *L. terrestris* hemoglobin subunits.

Estimates of the conformational free energy of unfolding, $\Delta G^\circ_{U,w}$, in neutral aqueous solutions, based on linear extrapolation of the denaturation data obtained with the different ureas dictated by eq 11 (Figure 6), give a $\Delta G^\circ_{U,w}$ value of 4.4 ± 0.3 kcal/mol that is not very different from the values of 3.1 and 3.4 kcal/mol obtained with the four-chain human and single-chain glycera oxyhemoglobin (E. R. Pandolfelli and T. T. Herskovits, unpublished results). These hemoglobin $\Delta G^\circ_{U,w}$ are on the low side of the values reported for other proteins, extrapolated by the same procedure. These values range from 5.8 for lysozyme, to 7.3–12.7 for horse heart cytochrome *c*, to 14.3 kcal/mol for sperm whale ferrimyoglobin (Puett, 1973; Knapp and Pace, 1974; Aune and Tanford, 1969).

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