Dissociation of Human Hemoglobin by the Ureas and Amides. Osmotic Pressure and Light Scattering Studies†

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ABSTRACT: The subunit dissociation of human hemoglobin by various ureas and amides has been investigated by osmometric and light scattering molecular weight methods. The effectiveness of these compounds as subunit dissociating agents is found to increase with increasing chain length or hydrocarbon content of the substituent alkyl groups. Nearly complete dissociation into half-molecules can be achieved before any pronounced changes are noticed in the physical properties of hemoglobin, signifying the unfolding of the intact $\alpha\beta$ subunits. The order of effectiveness of these two groups of reagent as both subunit dissociating agents and as denaturing agents, at higher concentrations of reagent, is found to be the same. The observed empirical correlations found between the dissociating effects and the relative hydrophobicities of these two series of compounds, as reflected by their binding constants, K_B, suggest that the nonpolar interactions at the $\alpha\beta$-contact sites of the hemoglobin subunits can be destabilized leading to increased subunit dissociation.

Various neutral salts are known to dissociate hemoglobin (Benhamou *et al.*, 1960; Rossi-Fanelli *et al.*, 1961; Benesch *et al.*, 1962; Guidotti, 1967; Kellett, 1971; Norén *et al.*, 1971; Thomas and Edelstein, 1972). In principle, by studying the effectiveness of various salts and reagents as subunit dissociating agents and the effects of these reagents on the solubility of amino acids and peptide model compounds one should be able to predict what sort of amino acid and peptide interactions are important for the maintenance of the intact tetrameric structure of hemoglobin (Kirshner and Tanford, 1964; Kawahara *et al.*, 1965) or any other subunit protein. This approach has been used by various workers (reviewed by Kauzmann, 1959; Von Hippel and Schleich, 1969; Tanford, 1970) in studies concerning the factors and forces that are important for the maintenance of the folded, native structure of proteins as well as the nucleic acids (Michelson, 1963; Herskovits and Harrington, 1972).

The effects of increasing hydrocarbon content of the urea, amide, and alcohol classes of denaturants on the conformation of single-chain globular proteins studied in the past 5 years in our laboratory (Herskovits and Jaillet, 1969; Herskovits *et al.*, 1970a–c) have now also been extended to similar studies on the single- and multi-chain hemoglobins (Elbaum *et al.*, 1973, 1974; Herskovits *et al.*, 1973). These studies in part also prompted the present investigation, dealing with the effects of the ureas and amides on the subunit structure of the four-chain human hemoglobin, examined by osmometric and light scattering molecular weight methods. The accompanying paper (Elbaum *et al.*, 1974) examines the effects of these two classes of compounds on the denaturation of the single-chain *Glycera dibranchiata* hemoglobin and four-chain human hemoglobin.

Experimental Section

Hemoglobin. Human hemoglobin was prepared from freshly drawn or spent defibrinated blood essentially according to Drabkin's (1946) procedure. The centrifuged erythrocytes were washed three times using 0.9% KCl solutions and lysed with 10:4 volume mixtures of distilled water and toluene in the cold followed by centrifugation to remove the cell debris and purification by passage through DEAE-Sephadex G-50 columns (Huisman and Dozi, 1965). Twice crystallized commercial hemoglobin (Schwarz/Mann) was purified by the same chromatographic procedure after conversion to the ferro state by passage through Sephadex G-25 columns charged with sodium dithionite (Dixon and McIntosh, 1967). All the hemoglobin solutions were stored at 4 ± 2° and were usually consumed within a week after preparation.

Hemoglobin Concentration. Protein concentration was determined spectrophotometrically on a Cary 14 recording spectrophotometer, using the per cent extinction coefficients of 9.04 for carboxyhemoglobin at 540 nm and 8.4 for deoxyhemoglobin at 555 nm. These values were based on the molar extinction coefficient of 4.6×10^4 and a mol wt of 64,450 daltons, at 540 nm for the cyanmet form of hemoglobin (Drabkin and Austin, 1935), obtained by conversion with Drabkin's reagent.

Reagents. All the ureas and amides were of the purest commercially available quality or spectral grade. With the exception of urea which was used without further purification (Schwarz/Mann Ultra Pure grade), all the solid ureas and amides were recrystallized from hot ethanol and were dried in a vacuum oven. 2,3-Diphosphoglycerate solutions were prepared by converting the pentacyclohexylammonium salt of the compound, purchased from Calbiochem, to the free acid form, using aqueous or buffered suspensions of Dowex 50W-X2 ion exchange resin (Benesch *et al.*, 1969).

Osmometry. Osmotic pressure measurements were made with a Wescon high-speed membrane osmometer utilizing Schleicher and Schuell B19 membranes. Before use the membrane compartment of the instrument was flushed with the appropriate solvent to be used and left to equilibrate overnight against the solvent at 25°. The performance of the instrument and the permeability of the Schleicher and Schuell

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TABLE I: Light Scattering Data of Carboxyhemoglobin and Solvent Densities of Various Urea and Amide Solutions.

Solvent	Refractive Index Increment $(\partial n/\partial c)_\mu$[a] 8 ± 2°	25 ± 1°	Refractive Index at 630 nm, n_{630}	Depolarization Ratio,[a] ρ	Cabannes factor,[a] $(6 + 6\rho)/(6 - 7\rho)$	Solvent Density at 25°
0.1 M KCl–0.02 M phosphate (pH 6.9)	0.194 ± 0.003	0.193 ± 0.003[b]	1.327	0.014[b]	1.031	1.0027
1.0 M urea	0.194	0.192	1.335	0.017	1.038	1.0198
1.0 M methylurea	0.190	0.191	1.337	0.024	1.052	1.0157
1.0 M ethylurea	0.188	0.190	1.339	0.027	1.060	1.0133
1.0 M propylurea	0.186	0.188	1.342	0.041–0.062[a]	1.09–1.145[a]	1.009
0.2 M butylurea	0.194	0.193	1.331	0.020	1.044	1.006
1.0 M 1,3-dimethylurea						1.011
0.5 M 1,3-diethylurea						1.004
0.25 M tetramethylurea						1.003
1.0 M thiourea						1.020
1.0 M formamide		0.192	1.328	0.0146	1.032	1.011
1.0 M acetamide		0.191	1.330	0.010	1.020	1.008
1.0 M propionamide		0.191	1.335	0.015	1.033	1.006
1.0 M butyramide		0.188	1.342	0.018	1.041	1.001

[a] Average values of three–five determinations; ranges of protein concentration 0.8–7 g/l. Only the values of propylurea showed any definite concentration dependence, with $\rho = 0.041$ at $c = 3.76$ g/l., $\rho = 0.054$ at $c = 2.5$ g/l., and $\rho = 0.062$ at $c = 0.849$ g/l. [b] The $(\partial n/\partial c)_\mu$ values for deoxyhemoglobin in the presence and absence of a 10 molar excess of diphosphoglycerate were 0.193 and 0.194 ml/g, respectively; the corresponding ρ value in the presence of diphosphoglycerate was 0.031.

membranes were checked by making measurements of well-characterized proteins. The data obtained on sperm whale myoglobin (Schwarz/Mann), bovine serum albumin (Pentex), and bovine thyroglobulin (Sigma) gave molecular weights of 16,900, 72,700, and 690,700, respectively, that are in good agreement with the literature values (Edsall *et al.*, 1950; Edsall, 1953; Klotz and Darnell, 1969).

Urea and amide solutions of hemoglobin were prepared volumetrically by diluting the given protein stock solution in 0.1 M KCl–0.02 M (pH 6.9) phosphate buffer with concentrated urea or amide solutions containing the same concentration of salt and buffer. Dialysates were prepared from the same solvents using the same urea or amide stock solutions. For the experiments with carboxyhemoglobin the protein solutions and dialysates were saturated with CO, followed by dialysis overnight in the cold. Cyanmethemoglobin solutions were prepared by the addition of five- to eightfold molar excess of Drabkin's reagent to both the protein solutions and the solvents. Measurements were made at 25°, with the instrument being calibrated using the final dialysates brought to room temperature.

Our osmotic pressure data were interpreted on the basis of eq 1 relating the measured osmotic pressure π and number average molecular weight, M_n

$$\pi/cRT = 1/M_n + B'c \tag{1}$$

where c is the concentration of the protein in grams per liter, R is the gas constant, T is the absolute temperature, and B' is the second virial coefficient representing the nonideality of the protein–solvent system as a function of protein concentration (Edsall *et al.*, 1950; Doty and Edsall, 1951). The measured osmotic pressure, π, is expressed in centimeters of solution, while R has a value of $84.68/d$, where d is the density of the solvent employed. Table I lists most of the urea and amide densities required for this density correction of R. For a subunit protein such as hemoglobin that dissociates into m subunits according to the equilibrium scheme, $P_l \rightleftharpoons mP_{l/m}$, the dissociation constant, K_{diss}, can be expressed as

$$K_{diss} = \frac{m^m \alpha^m c^{m-1}}{(1 - \alpha)(M_l)^{m-1}} \tag{2}$$

where M_l is the molecular weight of the undissociated protein (*i.e.*, 64,450 daltons per hemoglobin tetramer) and α is the fraction of protein dissociating. For hemoglobin tetramers dissociating into dimers $m = 2$ and for tetramers dissociating into monomers $m = 4$. The usual definition of the number average molecular weight, M_n (Tanford, 1961), with $n_4(1 - \alpha)$ representing the number of moles of undissociated hemoglobin of molecular weight M_4 and $(mn_4\alpha)$ representing the moles of dissociated species in the solution having a molecular weight, (M_4/m), gives us

$$M_n = \frac{n_4(1 - \alpha)M_4 + mn_4\alpha(M_4/m)}{n_4(1 - \alpha) + mn_4\alpha} = \frac{M_4}{[1 + (m - 1)\alpha]} \tag{3}$$

and in terms of the osmometrically determined molecular weights the estimates of α required to calculate K_{diss} (eq 2) can be expressed as

$$\alpha = \left\{\frac{1}{(m - 1)}\right\}\left(\frac{M_4}{M_n} - 1\right) \tag{4}$$

For a given concentration c, the experimentally determined (π/cRT) values have to be corrected for the nonideality, B', according to the expression

$$M_n = [\pi/cRT - B'c]^{-1} \tag{5}$$

The K_{diss} and B' values used in most of this study (Table II) were estimated essentially according to the method described by Guidotti (1967). In general, with estimates or trial values of K_{diss} for either the tetramer to dimer or tetramer to monomer mode of dissociation, the osmometric data can be fitted

TABLE II: Osmometric Results of Hemoglobin in Various Ureas and Amides.

Solvent	M_n at c = 4 g/l.[a]	% Dissociated at c = 4 g/l.[b]	Tetramer to Dimer 10^5K_{diss} (M)	$10^5B'$ (mol ml/g^2)
Carboxyhemoglobin				
0.1 M KCl–0.02 M phosphate (pH 6.9)	56,200	14	0.6	6
1.0 M urea	54,300	19	1	5
1.0 M methylurea	42,600	51	13	10
1.0 M ethylurea	38,800	66	30	15
1.0 M propylurea	38,000	70	40	15
0.2 M butylurea	42,000	53	15	10
1.0 M 1,3-dimethylurea	38,800	66	32	15
0.5 M 1,3-diethylurea	36,400	78	70	10
0.25 M tetramethylurea	38,000	70	40	10
0.1 M thiourea	51,300	26	2	6
1.0 M thiourea	38,800	70	40	10
0.15 M ethylthiourea	43,300	49	12	5
0.1 M butylthiourea	46,500	39	6	10
1.0 M formamide	42,600	52	14	5
1.0 M acetamide	42,100	53	15	10
1.0 M propionamide	36,900	75	56	10
1.0 M butyramide	34,000	90	180	15
Cyanmethemoglobin				
Water	55,200	17	0.9	5
2.0 M urea	53,800	20	1.1	4
2.0 M methylurea	49,000	32	4	4
1.0 M ethylurea	46,300	39	6.2	10
1.0 M propylurea	42,400	52	14	10

[a] Molecular weights based on smoothed π/cRT *vs.* c curves such as in Figures 2–5, and corrected for B'. [b] Per cent dissociation calculated by use of eq 4 (*i.e.*, $\alpha \times 100\%$).

using eq 6. The theoretical π/cRT values corresponding to

$$\left(\frac{\pi}{cRT}\right) = \left(\frac{1 + (m - 1)\alpha}{M_1}\right) + B'c \qquad (6)$$

different concentrations, c, are most readily obtained by assuming various α values and calculating c on the basis of eq 2.

Light Scattering. Measurements were made in a light scattering photometer of Brice's design (Brice *et al.*, 1950) made by Wood Mfg. Co. Due to strong absorpton of hemoglobin solutions in the conventional wavelength regions used for most light scattering investigations (*i.e.*, 436 and 546 nm), the instrument had to be modified by the manufacturer to allow measurements in the low absorbing red region. The modified instrument contains a 300-W tungsten lamp as the light source used in conjunction with a 630-nm interference filter to isolate monochromatic radiation at this wavelength and a photomultiplier tube (1P21 Hamamatsu Corp.) specially selected for good response characteristics in the red wavelength region. Dialyzed solutions and solvent were clarified by filtration through a double set of Millipore filters placed on top of one another. Metricel glass filters of 0.20 μ porosity and 25-mm diameters were found to give the most satisfactory results as far as removal of particulate matter. Square cells of

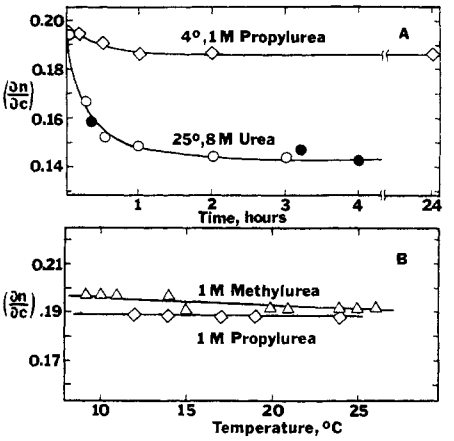

FIGURE 1: Effects of the length of dialysis (A) and temperature (B) on the refractive index increment, $(\partial n/\partial c)$, of CO-hemoglobin. All the solutions contained 0.1 M KCl and 0.02 M phosphate (pH 6.9) buffer. Protein concentration used ranged from 1.3 to 6.3 g/l.

24 × 24 × 70 mm capacity were used. The acceptability of solutions as judged by the relative absence of dust was determined by viewing the illuminated portion of the solutions with a mirror placed at low angles in relation to the emerging light beam, passing through the light scattering cell. Since the molecular dimensions of hemoglobin are less than 10% of that of the wavelength of light, the observed turbidities are independent of scattering angle. Consequently all measurements were made at 90°, relative to the angle of the incident beam (Doty and Edsall, 1951). Depolarization and absorption corrections were applied to all the calculated excess turbidities, τ, as described in the manual of the scattering instrument. Table I lists the depolarization ratios, ρ, obtained in various solvents and the corresponding Cabannes factors $[(6 + 6\rho)/(6 - 7\rho)]$. Comparable values of 1.04 and 1.07 have been reported for bovine serum albumin (Edsall *et al.*, 1950) and for lysozyme (Halwer *et al.*, 1951).

Urea and amide solutions were prepared by volumetric dilution using concentrated solutions of diluent and protein, both containing 0.1 M KCl and 0.02 M phosphate (pH 6.9) buffer, followed by overnight dialysis in the cold. As shown in Figure 1 dialysis equilibrium was usually reached after about 2 hr. Most of the solutions were separately dialyzed as described in the previous section. Deoxyhemoglobin solutions were prepared by bubbling nitrogen through both the solutions and solvents, followed by the addition of 2 mg/ml of sodium dithionite to both solutions. The solutions were filtered in a glovebag under nitrogen atmosphere and tightly covered with square pieces of parafilm. The stability of the solutions thus obtained was checked spectrophotometrically. Acceptable solutions had absorption ratios at 555–540 nm in the neighborhood of 1.24 (Benesch *et al.*, 1965) that remained unaltered for several hours. Light scattering measurements were usually made within 0.5 hr after filtering of the solutions. The same method was used to prepare deoxyhemoglobin solutions in the presence of diphosphoglycerate.

The light scattering results obtained were analyzed using the Debye equation where τ is the excess turbidity of the

$$H'c/\tau = 1/M_w + 2B'c \qquad (7)$$

protein solution over that of the dialysate, M_w is the weight average molecular weight, and H' is the usual light scattering constant which also contains the Cabannes depolarization correction, $H' = 32\pi^3n^2(\partial n/\partial c)_\mu^2/3N\lambda^4[(6 + 6\rho)/(6 - 7\rho)]$, where n is the refractive index of the solvent, $(\partial n/\partial c)_\mu$ is the

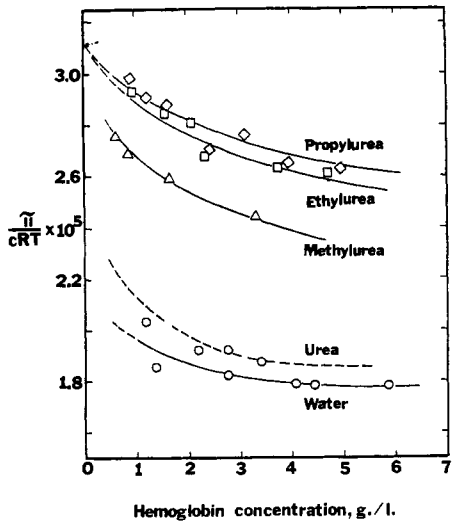

FIGURE 2: The effects of various 1.0 M ureas on the osmotic pressure data of CO-hemoglobin at 25°. All the solutions contain 0.1 M KCl and 0.02 M phosphate (pH 6.9) buffer.

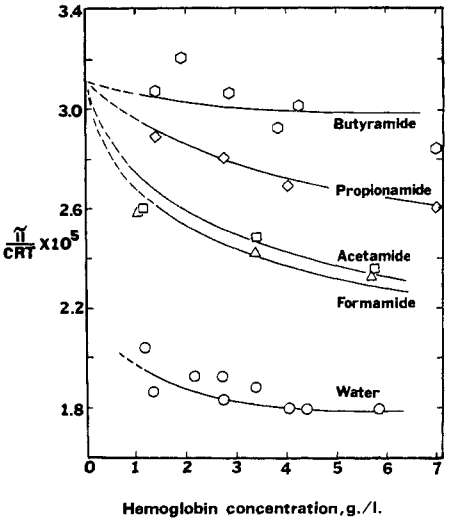

FIGURE 3: The effect of various 1.0 M amides on the osmotic pressure data of CO-hemoglobin at 25°. Solvent conditions the same as in Figure 2.

refractive index increment at constant chemical potential obtained with dialyzed solutions (Casassa and Eisenberg, 1964), N is Avogadro's number, and λ is the wavelength of light used for the scattering of light.

Based on the definition of the weight average molecular weight, $M_w = \Sigma n_i M_i^2 / \Sigma n_i M_i$, where n_i is the number of moles of ith species defined in the Osmometry section for the undissociated and dissociated subunits present in a given solution, we obtain the expressions

$$M_w = \frac{n_4(1-\alpha)M_4^2 + mn_4\alpha(M_4/m)^2}{n_4(1-\alpha)M_4 + mn_4\alpha(M_4/m)} = M_4\left[1 - \left(\frac{m-1}{m}\right)\alpha\right] \quad (8)$$

The fraction of tetramer dissociating, α, based on the observed light scattering molecular weight is equal to

$$\alpha = \left(\frac{m}{m-1}\right)\left[1 - \left(\frac{M_w}{M_4}\right)\right] \quad (9)$$

Noren *et al.* (1971) have obtained the same result cast in different form, and have concluded from this that light scattering measurements are less sensitive than osmotic pressure measurements to subunit dissociation. Combining eq 7 and 8 we obtain the general expression 10, analogous to eq 6, that can be used in conjunction with eq 2, the dissociation constant, to fit the experimental data with the best K_{diss} value. This equation is

$$\left(\frac{H'c}{\tau}\right) = \left\{\frac{1}{M_4\left[1 - \left(\frac{m-1}{m}\right)\alpha\right]}\right\} + 2B'c \quad (10)$$

To fit our light scattering data we have used the B' values based on the osmometric results (Table II) and have calculated the theoretical ($H'c/\tau$) curves by assuming fixed K_{diss} and varying α values to generate a set of c values (based on eq 2). From these curves both the mode of dissociation (*i.e.*, tetramer to dimer *vs.* tetramer to monomer) and the best K_{diss} can be readily assessed.

Differential Refractometry. The refractive index increments at constant chemical potential, $(\partial n/\partial c)_\mu$, and the refractive indices of the various urea and amide solutions at 630 nm were determined directly in the Wood light scattering photometer using the differential refractometer attachment made by the same manufacturer. The refractometer was calibrated using the KCl data of Kruis (1936). The interpolated Δn values at 630 nm of 1.4483×10^{-6} for 1.0794 g of KCl/100 ml and 5.2625×10^{-6} for 3.9969 g of KCl/100 ml at 25°, based on the Δn data reported for several wavelengths, were used. The cell constant, k, for the differential cell used for most of our measurements agreed to within 0.3% of the value based on the ratio magnification constant (Brice and Halwer, 1951) supplied by the manufacturer. Table I gives both the $(\partial n/\partial c)_\mu$ data obtained at both 8 ± 2° and 25 ± 1°, together with the refractive index data and the other constants used in light scattering and osmometry. Within experimental uncertainties of the measurements the $(\partial n/\partial c)_\mu$ values of hemoglobin in the various solvents of Table I seem to be unaffected by the change in temperature from 8 to 25°. On an average the $(\partial n/\partial c)_\mu$ values are approximately 0.002–0.008 ml/g lower in the 1 M urea and amide solutions listed than in the aqueous media. The latter values are in satisfactory agreement with the values of 0.192, in 0.05 M phosphate buffer, obtained at 632.8 Å by Noren *et al.* (1971). The experiments of Figure 1A indicate that dialysis equilibrium was usually reached after about 2–3 hr of magnetic stirring of the hemoglobin solutions dialyzed, provided the protein and the dialysates were carefully prepared by volumetric dilution (usually 1:4 volumes, respectively), using the same urea or amide solutions and aqueous solvents containing the same amount of KCl and buffer. Some turbidity was noted in the 25° dialysis experiments with 1 M methyl- and propylurea. For this reason most of our dialyses for light scattering and differential refractometry were carried out in the cold (4 ± 2°) for 16–24 hr. It is perhaps worth noting that Span and Lapanje (1973) have observed similar changes in refractive index increments to ours on dialysis of β-lactoglobulin and chymotrypsinogen A in 8 M urea solutions, with final $(\partial n/\partial c)_\mu$ values of 0.145 and 0.135 ml/g at 436 nm. Our equilibrium value for CO-hemoglobin in this solvent, shown in Figure 1A, is 0.143 ± 0.003 ml/g at 630 nm.

Optical Rotatory Dispersion (ORD) Measurements. Measurements were made in a Cary 60 recording spectropolarimeter. The mean residue rotations calculated, $[m]_\lambda$, were corrected for the refractive index of the solvent described in the accompanying paper and elsewhere (Elbaum *et al.*, 1974; Herskovits *et al.*, 1970a,c).

TABLE III: Subunit Dissociation and Estimates of Unfolding of Hemoglobin in Urea and Ethylurea Solutions, 0.1 M KCl–0.02 M Phosphate (pH 6.9).

Solvent	Osmotic Pressure Data[a]			ORD Data[b]	
	M_n[a]	% Dissociated[a] ($\alpha \times 100\%$)	Tetramer to Dimer $10^5 K_{diss}$	$[m']_{233}$	% Denatured[b]
	Cyanmethemoglobin, c = 0.24 g/l.[a]				
0 M urea	42,200	53	0.9	−9200[c]	0
2.0 M urea	40,800	58	1.0	−9200	0
3.0 M urea	40,000	61	1.4	−9200	0
4.0 M urea	38,400	68	2.0	−8800	5
5.0 M urea	34,700	86	8	−8500	7
6.0 M urea	26,000	(100)		−7900	15
7.0 M urea	24,000			−7200	
8.0 M urea	19,000			−6800	
	Carboxyhemoglobin, c = 0.47 g/l.[a]				
0 M ethylurea	47,400	36	0.6	−9060[c]	0
0.25 M ethylurea	39,800	62	3	−9060[c]	0
0.75 M ethylurea	37,700	74	6	−9060[c]	0
1.0 M ethylurea	33,600	92	30	−9060	0
2.0 M ethylurea	33,200	94	46	−8995	2

[a] Estimates of M_n and per cent dissociation obtained from Figure 4 and ethylurea data not shown, at protein concentrations of 0.24 and 0.47 g/l. [b] Estimates based on $[m']_{233}$ data using the equation of Chen *et al.* (1972). $[m]_{233} = -12{,}700 f_H - 2520$, where f_H represents the fraction of the protein in α-helical form and $[m]_{233}$ is the mean residue rotation divided by the dispersion factor, $3/(n^2 + 2)$, which is equal to 0.764 at 233 nm. [c] Data taken from smoothed $[m']_{233}$ *vs.* urea concentration curves of Elbaum *et al.* (1974). $[m']_{233}$ represent values from single ORD experiments.

Results

Osmometry. The data based on measurements of the osmotic pressure of CO-hemoglobin in various 1.0 M urea solutions are shown in Figure 2. In accordance with eq 1 the displacement of the $\pi/(cRT)$ *vs.* c curves to higher values of the ordinate indicates that there is increasing dissociation of the tetrameric structure of the protein with increasing alkyl chain length of the ureas. Similar effects with increasing hydrocarbon content and hydrophobicity have been obtained with the straight-chain amides shown in Figure 3. The trend in the molecular weight behavior of human hemoglobin due to the dissociating action of these two series of solutes is summarized in Table II. The results obtained with some of the thioureas are also included in this table, with all the dissociation constants, K_{diss}, based on the tetramer to dimer mode of dissociation and the experimental second virial coefficients, B', listed. At the urea and amide concentrations of 1 M and below, employed in these studies, no significant denaturation or unfolding of the hemoglobin subunits has been noted for any of solvents used. This is suggested by the absence of any significant changes in absorbance at the Soret region and the optical rotation at 233 or 265 nm (Elbaum *et al.*, 1973, 1974). Table III presents some of the osmometric and ORD results obtained on urea and ethylurea as a function of concentration of the dissociating agent. From the estimates of the fraction of dissociated and unfolded hemoglobin based on these measurements and listed in the second and last columns of this table, it is apparent that relatively little unfolding of the subunits is noted at the higher urea concentrations, where the hemoglobin has been largely dissociated to $\alpha\beta$ half-molecules. For example, in 5 M urea solutions 86% of the hemoglobin is dissociated at the concentration of the ORD experiments of 0.24 g/l. of hemoglobin, with estimates of only 7% denaturation. Our results are in close agreement with the conclusions of Steinhardt (1938) based on sedimentation and diffusion measurements on horse hemoglobin. In 4 M urea Steinhardt's value for the mol wt was 39,000. This value is nearly the same as our human hemoglobin value of 38,400. From the data of this table and also of Figure 4 it is apparent that above 5 M urea the hemoglobin chains start to unfold and further dissociation of $\alpha\beta$ dimer to single chains occurs. The osmometric molecular weights fall below the 32,230 value that should characterize the intact dimer.

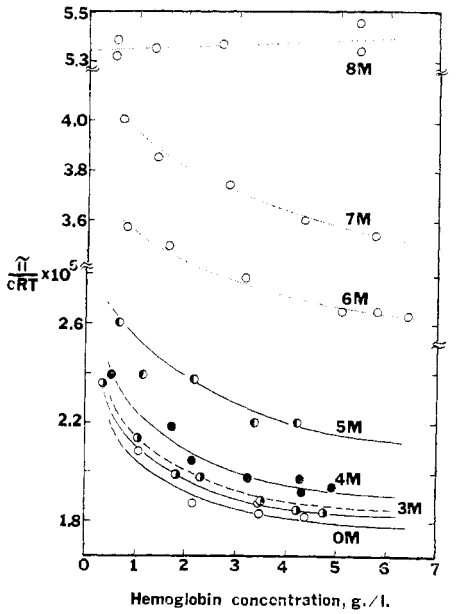

FIGURE 4: The effects of urea concentration on the osmotic pressure data of cyanmethemoglobin. Solvent conditions the same as in Figure 2.

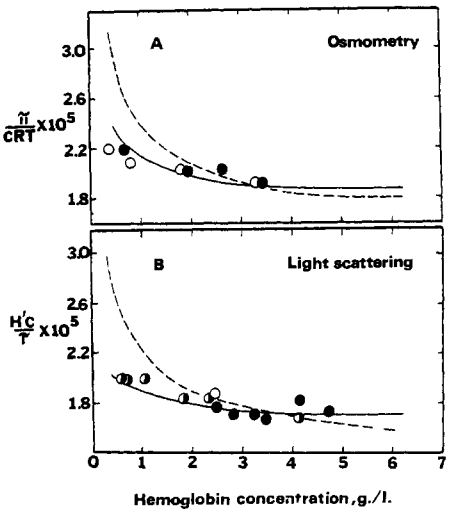

FIGURE 5: A comparison of the osmometric (A) and light scattering (B) data of 1.0 M urea solutions of CO-hemoglobin: (○) 25 ± 1°; (◑) 8 ± 2°; (●) 25°, in the presence of 0.01 M KCNO. Other solvent conditions are the same as in Figure 2. The solid lines represent a theoretical tetramer to dimer dissociation equilibria, with the best $K_{diss} = 1 \times 10^{-5}$ for the osmotic pressure data and 0.8×10^{-5} for the light scattering data, fitted as described in the text. The dashed lines represent theoretical curves for tetramer to monomer dissociation with $K_{diss} = 2 \times 10^{-14}$ for the osmometric and 1×10^{-15} for the light scattering data.

All of our data except those obtained in 5–8 M urea solutions could best be fitted with dissociation constants characterizing the tetramer to dimer splitting of native hemoglobin. The solid lines drawn through all the related data sets of Figures 2–5 were based on this model of hemoglobin dissociation. Figure 5 shows both the tetramer to dimer and tetramer to monomer modes of dissociation fitted according to eq 6 and 10 and the B' values of Table II. It is clear that the tetramer to monomer curves generated by a single K_{diss} value, and represented by the dashed lines, do not describe adequately the concentration dependence of the osmometric and light scattering behavior of hemoglobin in urea solutions. The data of this figure also include results obtained in 1 M urea solutions containing 0.01 M potassium cyanate. The fact that overnight dialysis in the presence of this reagent had no significant effects on the apparent molecular weights and dissociation of hemoglobin (data represented by filled circles) suggests that possible carbamylation of ε-amino and N-terminal residues of hemoglobin by trace amounts of cyanate found in urea solutions (Stark *et al.*, 1960) is not the cause of the observed dissociation of hemoglobin in urea solutions. Cerami and Manning (1971) have found that cyanate inhibits the sickling of hemoglobin S.

The overall equilibrium for the reaction between hemoglobin and the dissociating agent, D, leading to the splitting of hemoglobin to $\alpha\beta$ dimers can be expressed as

$$Hb_4 + nD \rightleftharpoons 2Hb_2D_{n/2} \qquad (11)$$

with the equilibrium constant

$$K_n = \frac{[Hb_2D_{n/2}]^2}{[Hb_4][D]^n} = \frac{K_{diss}}{[D]^n} \qquad (12)$$

Since the dissociation constant, K_{diss}, is obtained from osmometric and light scattering measurements, the order of the dissociation reaction with respect to the dissociating agent, n, can be obtained from the slope of log K_{diss} *vs.* log [D] plots. Figure 6 shows such plots for the ethylurea dissociation of carboxyhemoglobin and the urea dissociation of cyanmethemoglobin. Least-squares analysis of the data of this figure gave $n = 1.6 \pm 0.4$ for ethylurea and 2.0 ± 0.6 for urea dissociation. It is perhaps significant in relation to our n values that the NaCl data of Thomas and Edelstein (1972) on unliganded hemoglobin gave us n values of 1.4 ± 0.4 for this salt.

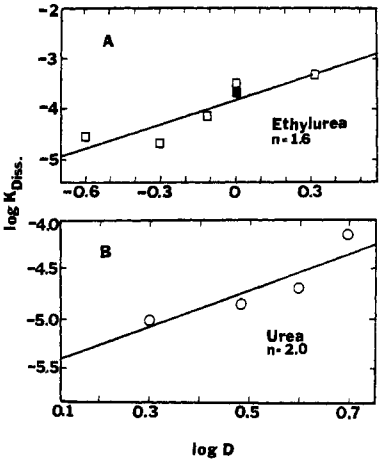

FIGURE 6: Log K_{diss} *vs.* log [D] curves for CO-hemoglobin in 0.25–2.0 M ethylurea solutions (A) and for cyanmethemoglobin in 2–5 M urea solutions (B), plotted according to eq 12 (using the osmometric K_{diss} data). The solid lines represent least-squares fit of data with slopes $n = 1.6 \pm 0.4$ for the ethylurea and $n = 2.0 \pm 0.6$ for the urea data.

Light Scattering. Figures 7 and 8 show some of the light scattering data obtained with the urea and amide series of solutes. The same trend of increasing dissociation of hemoglobin and upward displacement of the curves, with increasing hydrophobicity of the series, is seen as in the case of the osmometric results (Figures 2 and 3). Table IV presents a summary of our light scattering studies with these two series of solutes. Again the dissociation constants, K_{diss}, presented are based on the tetramer to dimer mode of dissociation, that were fitted according to the method described in the Experimental Section, using eq 10. Figure 5B presents and compares the tetramer to dimer and tetramer to monomer fit of the experimental results obtained with 1 M urea solutions, the latter curve having been drawn with the dashed line. The molecular weights of proteins determined by the light scattering technique tend to be on the high side (Edsall *et al.*, 1950; Doty and Edsall, 1951; Edsall, 1953; Norén *et al.*, 1971) when compared

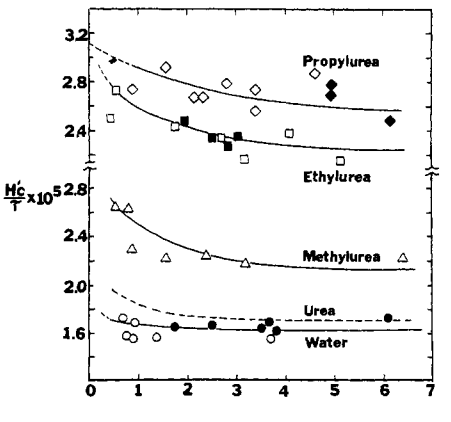

FIGURE 7: The effects of various 1.0 M urea on the light scattering of CO-hemoglobin. Open symbols represent 25 ± 1° data; closed symbols represent 8 ± 2° data. Solvent conditions are the same as in Figure 2.

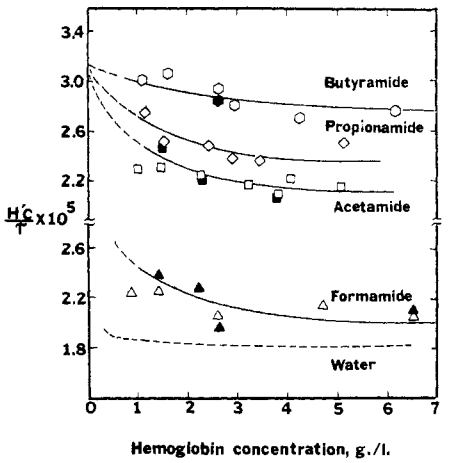

FIGURE 8: The effects of various 1.0 M amides on the light scattering of CO-hemoglobin. Open symbols represent 25 ± 1° data; closed symbols represent 8 ± 2° data. Dashed line represents data in aqueous salt solutions shown in Figure 7. Solvent conditions are the same as in Figure 2.

to molecular weight estimates based on other techniques. This is due to the problem of clarification and the scattering of residual dust or other particulate matter, and also to the greater sensitivity of this technique to aggregation of proteins, produced by the filtration and manipulation of the solutions required. For this reason our estimates of the K_{diss} listed with the various ureas and amides were based on both the theoretical tetramer mol wt of 64,450 daltons and our average experimental value of 68,900 daltons, obtained with deoxyhemoglobin in the presence and absence of a 10 molar excess of diphosphoglycerate. Least-squares analysis of the data obtained on deoxyhemoglobin solutions containing 2 mg/ml of sodium dithionite gave molecular weights of 73,000 ± 6000 and 64,700 ± 1600 daltons, respectively (Elbaum, 1974). The assumption made with the latter molecular weights is that subunit dissociation is practically absent at the 1-6 g/l. hemoglobin concentrations used in these experiments. Thomas and Edelstein (1972) have reported recently that the dissociation constant of deoxyhemoglobin in 0.1 M phosphate (pH 7) is 3×10^{-12} M.

Thermodynamic Parameters and Related Data. Table V presents a summary of the standard free energies of dissociation, ΔF°_D, of human CO-hemoglobin into half-molecules of $\alpha\beta$ dimers (Kawahara *et al.*, 1965; Guidotti, 1967; Edelstein *et al.*, 1970). The ΔF°_D values listed for the various urea and amide solutions have been calculated using both the osmometrically determined K_{diss} constants (Table II) and the related constants based on the light scattering data (Table IV), according to the equation

$$\Delta F^\circ_D = -RT \ln K_{diss} \quad (13)$$

In order to assess the number of newly exposed peptide units resulting from the dissociation of hemoglobin due to the interactions of the disrupted contact sites and the dissociating or disrupting reagent, we have also listed the differences in ΔF°_D in the presence and absence of the dissociating ureas and amides, and apparent number of binding sites, N, exposed using the equations of Schellman (1955) and Peller (1959) (eq 14), the assumption being that the free-energy

$$\Delta F_{B,N} = -NRT \ln (1 + K_B a_D) \quad (14)$$

differences arise as a result of these interactions between the exposed contact sites and the dissociating reagent. In eq 14 K_B is the binding constant between the dissociating agent and the average peptide unit exposed and a_D is the activity of the dissociating agent, *i.e.*, $c_D\gamma_D$. For weak binding with $K_B a_D \ll 1$, expansion of the logarithmic term and the activity coefficient, γ_D, taken as one leads to the simplified expression

$$\Delta F_{B,N} = -NRTK_B c_D \quad (15)$$

The standard free energy of dissociation, ΔF°_D, may be expressed as

$$\Delta F^\circ_D = \Delta F^\circ_{D,w} + \delta\Delta F^\circ_D \quad (16)$$

where $\Delta F^\circ_{D,w}$ represents the free energy of dissociation in aqueous salt solution and $\delta\Delta F^\circ_D$ the contribution to the free-energy change resulting from the transfer of the exposed peptide and amino acid side chains, resulting from the dissociation of the protein, from aqueous solution to solutions containing the disrupting reagent (Tanford, 1964). The $\delta\Delta F^\circ_D$ term in eq 16 may be expressed as the sum of the free

TABLE IV: Light Scattering Results of Carboxyhemoglobin in Various Ureas and Amides.

Solvent	M_w at c = 4 g/l.[a]	% Dissociation at c = 4 g/l.[b]		Tetramer to Dimer K_{diss}, $M \times 10^5$	
		M_4 = 64,450	M_4 = 68,900	M_4 = 64,450	M_4 = 68,900
		Ureas			
0.1 M KCl–0.02 M phosphate	62,600	6	18	0.1	0.9
1.0 M urea	59,500	16	28	0.8	3
1.0 M methylurea	47,400	52	62	14	24
1.0 M ethylurea	45,900	58	66	20	29
1.0 M propylurea	39,200	78	86	70	123
0.2 M butylurea	54,900	30	40	3	6
		Amides			
1.0 M formamide	48,800	49	58	12	19
1.0 M acetamide	47,600	52	62	14	23
1.0 M propionamide	43,500	66	74	32	50
1.0 M butyramide	36,800	86	93	130	287

[a] Molecular weights taken at c = 4 g/l. from smooth $H'c/\tau$ *vs.* c curves of Figures 7 and 8 and corrected for B'. [b] Per cent dissociation calculated by use of eq 9 (*i.e.*, $\alpha \times 100\%$).

TABLE V: Thermodynamic Data for Tetramer to Dimer Dissociation of CO-Hemoglobin by Various Ureas and Amides.

Solvent	Osmometry		Light Scattering		$(\Delta F°_{D,w} - \Delta F°_D)$[a] (kcal/mol)	10^2K_B[b]	ΔF_B[c] (cal/mol)	N[d]
	K_{diss} (10^5 M)	$\Delta F°_D$ (kcal/mol)	K_{diss} (10^5 M)	$\Delta F°_D$ (kcal/mol)				
0.1 M KCl–0.02 M phosphate	0.6	7.1	0.1	8.2				
1.0 M urea	1.0	6.8	0.8	7.0	0.75	3.2	−18.6	40
1.0 M methylurea	13	5.3	14	5.2	2.4	4.2	−24.3	99
1.0 M ethylurea	30	4.8	20	5.0	2.75	6.08	−34.9	79
1.0 M propylurea	40	4.6	70	4.3	3.2	10.8	−61.0	52
0.2 M butylurea	15	5.2	3	6.2	1.95	23.2	−26.2	75
1.0 M formamide	14	5.3	12	5.3	2.35	2.1	−12.3	191
1.0 M acetamide	15	5.3	14	5.3	2.35	3.11	−17.9	132
1.0 M propionamide	56	4.4	32	4.8	3.05	4.98	−28.6	107
1.0 M butyramide	~180	3.7	130	4.1	3.75	9.69	−54.8	70

[a] Average values of osmometric and light scattering parameters. [b] Data taken from accompanying paper (Elbaum *et al.*, 1974), with $K_B = K_{H\Phi} + K_P$ and $K_{H\Phi} = 1.01$, 2.88, 7.59, and 20 × 10^{-2} for methyl, ethyl, propyl, and butyl group contributions, and $K_P = 3.2$ and 2.1 × 10^{-2} for the $NH(CO)NH_2$ and the $CONH_2$ groups of the ureas and amides. [c] Calculated by use of the equation $\Delta F_B = -RTK_Bc_D$. [d] $(\Delta F°_{D,w} - \Delta F°_D)$ divided by $-\Delta F_B$ values, according to eq 18, where $\Delta F_B = -RTK_Bc_D$.

energies of transfer of the individual groups, $\Sigma\Delta f_{t,i}$, that are exposed on the dissociated half-molecules but do not include the surface groups of the undissociated, tetrameric protein, or alternatively as the sum of the free energy of binding of the dissociating reagent to the N exposed sites, $\Delta F°_{D,N}$, given by eq 15. Thus combining eq 15 and 16 gives for $\Delta F°_D$

$$\Delta F°_D = \Delta F°_{D,w} - NRTK_Bc_D \quad (17)$$

that can be used to estimate the average number of exposed binding sites N

$$N = (\Delta F°_{D,w} - \Delta F°_D)/RTK_Bc_D \quad (18)$$

The N values calculated for the dissociation of hemoglobin by the ureas and amides listed in the last column of Table V were calculated using eq 18. The K_B values used for these calculations, taken from the literature (Herskovits *et al.*, 1970b; Elbaum *et al.*, 1974), are also listed in this table.

Discussion

The most significant finding of the present investigation is that both the urea and amide series of denaturants are also very effective subunit dissociating agents for human hemoglobin. Moreover, the effects of the various ureas and amides on the osmometric and light scattering molecular weights of hemoglobin investigated (Tables II and IV) indicate increasing potency of subunit dissociation with increasing chain length or hydrocarbon content of these two groups of solutes. The same order of effectiveness has been found for these series as denaturing agents for single- and multi-chain hemoglobins (Herskovits *et al.*, 1973, Elbaum *et al.*, 1973, 1974) and other globular proteins (Herskovits *et al.*, 1970b,c). Related to the effects of the ureas and amides on the quaternary structure of hemoglobin it is important to note that nearly complete dissociation into half-molecules can be achieved (Table III) before any pronounced changes are noticed in the optical rotatory dispersion or the Soret absorption, signifying the unfolding of the intact hemoglobin subunits (Elbaum *et al.*, 1973, 1974). It is also important to note that relatively good agreement is obtained between the osmometric results reported in our earlier communications (Elbaum *et al.*, 1973) and the light scattering data (Tables II and IV). For example, the number-average and weight-average molecular weights obtained by these two techniques for hemoglobin solutions of 4 g/l. in 0.1 M KCl, 0.02 M phosphate (pH 6.9), and 1.0 M urea, propylurea, or propionamide are: 56,200 and 62,500, 54,300 and 59,500, 38,000 and 39,200, and 36,900 and 43,500 daltons, respectively. The progressive increase in subunit dissociation represented by these molecular weight estimates are respectively 14 and 6%, 19 and 16%, 70 and 78%, and 75 and 66% (Tables II and IV). Correspondingly satisfactory agreement is obtained between the dissociation constants, K_{diss}, for the various ureas and amides (Table VI), considering the experimental uncertainties inherent in these two methods (Doty and Edsall, 1951; Edsall, 1953). Kawahara *et al.*, (1965) have studied the dissociation of human hemoglobin

TABLE VI: Comparison of Experimental and Calculated Dissociation Constants, K_{diss}, of Carboxyhemoglobin in Various Ureas and Amides.

Solvent	10^2K_B[a]	10^5K_{diss}[b] Osmometry	Light Scattering	Calculated Eq 22 and 23
	Ureas			
1.0 M urea	3.2	1	0.8	6.1
1.0 M methylurea	4.2	13	14	10.6
1.0 M ethylurea	6.08	30	20	22
1.0 M propylurea	10.8	40	70	70
0.2 M butylurea	23.2	15	3	13[c]
	Amides			
1.0 M formamide	2.1	14	12	8.8
1.0 M acetamide	3.11	15	14	19
1.0 M propionamide	4.98	56	32	50
1.0 M butyramide	9.69	180	130	188

[a] Data taken from the accompanying paper, Table IV (Elbaum *et al.*, 1974). [b] The light scattering and osmometric constants are from Tables II and IV. [c] Calculated using eq 32.

by urea and various salts and have concluded that about 40 polypeptide groups are exposed as a result of subunit dissociation and are found to interact with the disruptive reagent, urea. These estimates were obtained by considering the effects of the free energy of transfer, $\Delta f_{t,i}$, of all the newly exposed groups (from aqueous solutions to solutions containing the dissociating reagent) on the standard free energy of dissociation of the protein into half-molecules

$$\Delta F^{\circ}_{D} = \Delta F^{\circ}_{D,w} + \Sigma \Delta f_{t,i} \quad (19)$$

where $\Delta F^{\circ}_{D,w}$ represents the standard free energy of dissociation in aqueous salt solutions in the absence of dissociating agent. Our own estimates based on the Schellman (1955) and Peller (1959) formalism (*i.e.*, eq 18 of the Results), with the binding term $NRTK_{B}c_{D}$ replacing the free-energy transfer term $\Sigma \Delta f_{t,i}$ summed over all the newly exposed groups resulting from the dissociating of the tetrameric protein, gave essentially the same estimates of groups that can interact with urea. These estimates for N, listed in Table V, are larger for the higher ureas and the amides, but seem to indicate no clear-cut trend with increasing hydrophobicity of these two classes of reagents. In relation to these calculations it is important to note that the required K_{B} values for the various ureas and amides studied were derived from denaturation and free-energy transfer data, on the assumption that the polar and nonpolar contributions to the binding constants are additive functions of the group values of the binding constant (Herskovits *et al.*, 1970b; Elbaum *et al.*, 1974)

$$K_{B} = K_{H\Phi} + K_{P} \quad (20)$$

where $K_{H\Phi}$ represents the nonpolar contribution of the alkyl groups and K_{P} the polar $HN(CO)NH_2$ and $CONH_2$ contributions to the average urea- and amide-peptide group interactions.

The crystallographic structure of hemoglobin (Perutz *et al.*, 1968) reveals two distinct pairs of surfaces of contact between the folded α and β chains, that consist of 34 and 19 amino acids residues per each contact. Unless compensating changes accompany the splitting of hemoglobin into symmetrical $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers, dissociation would result in the splitting and exposure of the contacts $\alpha_1\beta_2$ and $\alpha_2\beta_1$ comprised of twice 19 or a total of 38 amino acid residues.[1] In addition, two polar contacts (salt bridges) between like chains seem to be also split as a result of subunit dissociation. It is significant that the probable number of groups that is exposed in the course of dissociation, and can interact with the disruptive reagent, is about the same as our lower estimate obtained with urea (Table V).

The observed changes in the binding constants and the dissociation constants of hemoglobin given in Table V suggest that there should be a close correlation between these two sets of constants. An empirical or semiempirical relationship between the dissociation constants, K_{diss}, and the binding constants, K_{B}, should permit us to predict the effect of a dissociating agent, assuming that the group contributions of the reagent are additive. We find that a reasonable estimate of the dissociation constants can be obtained using the relationship

$$K_{diss} = [\text{const}]K_{B}^{2}[D]^{2} \quad (21)$$

For the dissociation of CO-hemoglobin by the 1.0 M ureas we obtain the expression

$$K_{diss} = 6 \times 10^{-2}K_{B}^{2} \quad (22)$$

and for the dissociation by the 1.0 M amides we obtain

$$K_{diss} = 2 \times 10^{-1}K_{B}^{2} \quad (23)$$

Table VI presents a comparison of the osmometric and light scattering dissociation constants with the values predicted by these two empirical equations, 22 and 23. The exponential factor of 2 is suggested by the log K_{diss} *vs.* log [D] plot for urea, shown in Figure 6.

The standard free-energy expression 19 suggests that the dissociation of hemoglobin in any solvent may be viewed as a consecutive set of reactions, characterized by a dissociation of the tetramer into two dimers followed by binding or association of the newly exposed amino acid sites with the dissociating agent. Consequently, the equilibrium should be shifted toward the right, that is, toward the dissociated half-molecules. The equilibrium for this process may be expressed as

$$Hb_4 + 2iD \overset{K_{D,w}}{\rightleftharpoons} 2Hb_2 + 2iD \overset{K_{D}}{\rightleftharpoons} 2(Hb_2D_i) \quad (24)$$

where

$$K_{D,w} = [Hb_2]^2/[Hb_4] \quad (25)$$

and

$$K_{D} = \{[Hb_2D_i]/([Hb_2][D_i]^i)\}^2 \quad (26)$$

For N' equivalent and independent binding sites per hemoglobin dimer, the association constant for the dimer–urea or dimer–amide reaction is given as (Klotz, 1953; Simpson and Kauzmann, 1953; Schellmann, 1955)

$$K_{D} = \left\{\frac{N'!}{(N'-i)!i!}K^{i}\right\}^{2} \quad (27)$$

where the average number of urea or amide molecules associated with each dimer is

$$\langle i \rangle = \left(\frac{K[D]}{1 + K[D]}\right)N' \quad (28)$$

with K representing the intrinsic binding or association constant with the average binding site and i is the number of urea or amide molecules that associate with each dimer. For the overall reaction in any dissociating solvent, expressions 24–27 lead to the expression

$$Hb_4 + 2iD \overset{K_{HbD}}{\rightleftharpoons} 2(Hb_2D_i) \quad (29)$$

where the equilibrium constant, K_{HbD}, and the related dissociation constant, K_{diss}, are equal to

$$K_{HbD} = K_{D,w}K_{D} = K_{D,w}\left\{\frac{N'!}{(N'-i)!i!}\right\}^{2} K^{2i} \quad (30)$$

$$K_{diss} = K_{HbD}[D]^{2i} = K_{D,w}[D]^{2i}\left\{\frac{N'!}{(N'-i)!n!}\right\}^{2} K^{2i} \quad (31)$$

If we equate the intrinsic binding constant K with the binding constants, K_{B}, that characterize the urea or amide interactions with the average peptide unit, and take $K_{D,w} = 5 \times 10^{-6}$ M (based on our osmotic pressure and light scattering

[1] According to Perutz *et al.* (1968) the smaller $\alpha_1\beta_2$ unsymmetrical contact contains one clearly discernible hydrogen bond between the side chains of aspartic acid-94α and asparagine-102β, and another possible hydrogen bond between the side chains of threonine-41α and histidine-97β. All the other interactions appear to be nonpolar. In order of decreasing hydrophobicity the number and kind of amino acid residues in this contact area most likely to be split as a result of dissociation (Kawahara *et al.*, 1965; Guidotti, 1967; Perutz *et al.*, 1968) are as follows: 1 Trp, 2 Tyr, 1 Leu, 3 Val, 1 His, 2 Thr, 2 Pro, 2 Arg, 1 Gln, 1 Asn, 1 Glu, and 2 Asp.

estimates of Tables II and IV and the more recent literature values (Guidotti, 1967)), and $i = 1.0$, eq 31 becomes

$$K_{diss} = 5 \times 10^{-6}[D]^2 K_B^2 (N')^2 \quad (32)$$

With $N' = 110$ for the ureas and $N' = 200$ for the amides, this equation is reduced to the simple form of eq 22 and 23, obtained experimentally.

Unfortunately, the N' values based on these equations are about 3–5 times larger than the corresponding values obtained by eq 18 (Table V). Also, the apparent order of the reaction, n (*i.e.*, $n = 2i$), for the higher ureas may not be exactly 2. For example, the ethylurea data are characterized by an n value of 1.6 ● 0.4 (Figure 6). Questions related to these problems are presently being investigated in our laboratory.[2] In addition, there could be other complicating factors arising from small structural differences in the $\alpha\beta$ half-molecules, as compared to the parent tetramer of hemoglobin, suggested by Guidotti (1967). Consequently, only semiquantitative predictions should be expected in any correlations of the observed dissociation effects of the ureas and amides and their relative hydrophobicities, as suggested by eq 22, 23, and 31.

Our findings clearly suggest that the nonpolar interactions at the $\alpha\beta$-contact sites of the hemoglobin subunits can be destabilized, leading to increased subunit dissociation with increasing hydrocarbon content of these two series of compounds. The desickling effects of urea administered to patients during the crisis phase of sickle-cell anemia have been attributed (Nalbadian *et al.*, 1970) to the weakening of the hydrophobic valine to valine contacts (Murayama, 1966) in the associated, sickle-form of hemoglobin S. Unless the beneficial effects of urea treatment are entirely due to the modification of hemoglobin by the cyanate (Cerami and Manning, 1971) present in urea solutions (Stark *et al.*, 1960), our results would suggest increased dissociation and desickling with the more hydrophobic, alkyl-substituted ureas.

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[2] A more exact formulation of the reaction between protein and the dissociating agent represented in eq 24 leads to a K_{diss} of the form (Schellman, 1955)

$$K_{diss} = K_{D,w}\left\{\sum_{i=0}^{N'}\left[\frac{N'!}{(N'-i)!i!}\right](K_B[D])^i\right\}^2 \quad (31a)$$

The evaluation of any thermodynamic parameter base on this formulation is cumbersome and difficult to apply. Partly for this reason the more approximate but simpler statistical factor, $N'!/(N'-i)!i!$ of eq 27, not containing the summation term of eq 31a, is usually employed (Klotz, 1953; Simpson and Kauzman, 1953; Schellman, 1955). Applied to eq 31a, the bionomial theory of expansion leads to

$$K_{diss} = K_{D,w}(1 + K_B[D])^{2N'} \quad (31b)$$

with the logarithmic form of this equation multiplied by $-RT$ to

$$\Delta F_{B,N} = -RT \ln (K_{diss}/K_{D,w}) = -2N'RT \ln (1 + K_B[D]) \simeq -2N'RTK_B[D] \quad (31c)$$

Recalling that $N = 2N'$, eq 31c is equivalent to eq 15 of the text. Qualitatively the higher estimates of N' deduced from eq 32 than from eq 18 are consistent with the retention of only one term, $i = 1$, in the summation of eq 31a. We are grateful to one of the reviewers for bringing this fact to our attention and also for this alternative derivation of eq 14. However, with regard to our data of Figure 6, we find that the log K_{diss} *vs.* log [D] plots, based on eq 12 and 32, give a somewhat better linear fit than the corresponding log $(K_{diss}/K_{D,w})$ *vs.* [D] plots, based on eq 31c.

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Denaturation of Human and *Glycera dibranchiata* Hemoglobins by the Urea and Amide Classes of Denaturants†

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ABSTRACT: The effects of both ureas and amides on the native conformation of the four-chain human hemoglobin and the single-chain component of *Glycera dibranchiata* hemoglobin were investigated by spectral and optical rotatory dispersion methods. Both techniques give similar transition profiles with essentially the same midpoints, S_m, and broadness for the various transitions. The midpoints of the denaturation transition as a function of urea and amide concentrations were analyzed according to the equations of Peller and Flory with appropriate binding constants and Setschenow constants calculated from free-energy transfer data and solubilities of *N*-acetyl-L-tryptophan ethyl ester. The same order of increasing effectiveness of the denaturant with increasing hydrocarbon content is exhibited by both hemoglobins. The denaturation of these hemoglobins in these two classes of solutes is found to be closely similar in behavior to that of other globular single-chain proteins.

At low to moderate solute concentrations both the urea and amide classes of denaturants cause the dissociation of human hemoglobin to half-molecules, consisting of $\alpha\beta$ dimeric species, without any pronounced alterations in the conformation of the protein (Elbaum *et al.*, 1973). At higher urea and amide concentrations one should expect that the denaturing action of these two groups of solutes should lead to the unfolding of the hemoglobin subunits and that the order of effectiveness as denaturing agents should be related to their relative hydrophobicities predicted by the equations of Peller (1959) and Flory (1957). Our previous studies on single-chain proteins have shown that hydrophobic interactions exert an increasingly greater stabilizing effect on the conformation of the unfolded or denatured form of proteins with increasing chain length or hydrocarbon content of the denaturant (Herskovits and Jaillet, 1969; Herskovits *et al.*, 1970a–c).

The findings of the accompanying study (Elbaum and Herskovits, 1974), showing that the effectiveness of the ureas and amides as subunit dissociating agents for hemoglobin are related to their relative hydrophobicities, prompted the denaturation study presented in this paper. We have examined the effects of the ureas and amides on both the absorbance in the Soret region and the optical rotatory dispersion in the far-ultraviolet region, related to the folding of the polypeptide chains, of the four-chain human hemoglobin species and the single-chain hemoglobin component of the blood of the worm, *Glycera dibranchiata*.

Experimental Section

Materials. The single- and multi-chain hemoglobin components of the bloodworm, *Glycera dibranchiata*, were isolated and prepared by Sephadex G-75 chromatography in the cold, essentially according to the method of Seamonds *et al.* (1971a,b). This species of live worms was obtained from Maine Bait Co., Newcastle, Maine, *via* air express. The human hemoglobin preparations and the reagents and solvents employed are described in the accompanying paper (Elbaum and Herskovits, 1974).

Methods. Absorbance and optical rotatory dispersion (ORD) measurements were made in Cary 14 and Cary 60 recording instruments. Hemoglobin concentrations were determined spectrophotometrically using the molar extinction coefficients per heme group of 1.2×10^4 at 545 nm for *Glycera* and 1.25×10^4 at 540 nm for human hemoglobin, after conversion of both hemoglobin species to the cyanomet form by use of Drabkin's reagent (Seamonds *et al.*, 1971a; Antonini, 1965). These extinction values correspond to 2.32×10^5 at 422 nm and 1.91×10^5 at 419 nm for the carbonmonoxy form of these two hemoglobins (Seamonds *et al.*, 1971a). The ORD data are reported as mean residue rotation, $[m']_\lambda = \alpha M_0/(cl)[3/(n_\lambda^2 + 2)]$, where α is the observed rotation at wavelength λ, c is the protein concentration in grams per 100 ml, l is the path length of the cell used in decimeters, and n_λ is the

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