Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1961), *J. Biol. Chem. 236*, 341.

Schellman, J. A. (1955), *C. R. Trav. Lab. Carlsberg 29*, 230.

Simpson, R. B., and Kauzmann, W. (1953), *J. Amer. Chem. Soc. 75*, 5139.

Span, J., and Lapanje, S. (1973), *Biochim. Biophys. Acta 295*, 371.

Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.

Steinhardt, J. (1938), *J. Biol. Chem. 123*, 543.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley.

Tanford, C. (1964), *Brookhaven Symp. Biol. 17*, 154.

Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.

Thomas, J. O., and Edelstein, S. J. (1972), *J. Biol. Chem. 247*, 7870.

Von Hippel, P. H., and Schleich, T. (1969), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 417.

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

Danek Elbaum, Emilia R. Pandolfelli, and Theodore T. Herskovits*

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

---

† From the Department of Chemistry, Fordham University, New York, New York 10458. *Received September 4, 1973.* This work was supported by Grant HL-14453 from the Heart and Lung Institute of the National Institutes of Health, U. S. Public Health Service. A preliminary report of this work was presented at the 1973 Meeting of the Federation of American Societies for Experimental Biology (Elbaum *et al.*, 1973).

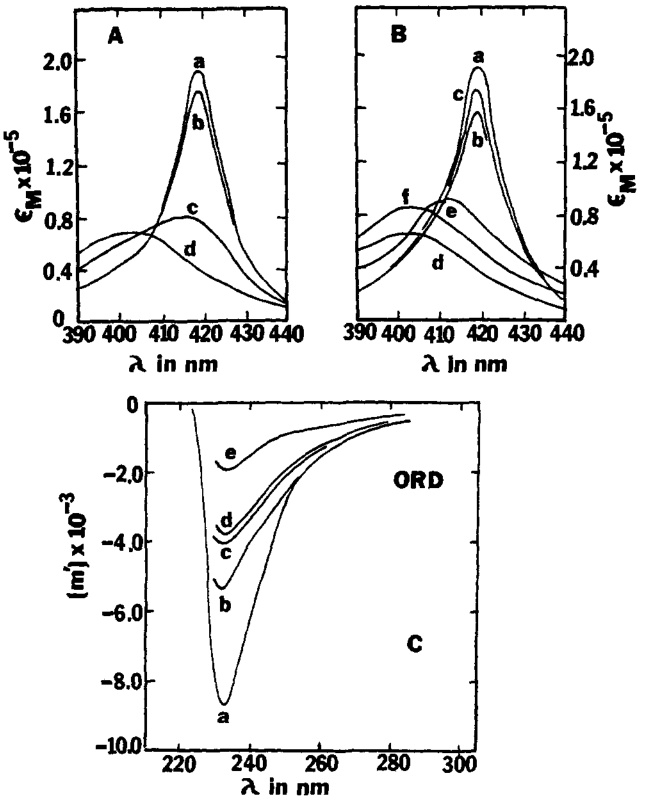

FIGURE 1: The denaturing effects of the ureas on the absorbance in the Soret region and the optical rotatory dispersion of human CO-hemoglobin: (A) curve a, water (native protein); curve b, 3 M ethylurea; curve c, 4.5 M ethylurea; curve d, 5–7 M ethylurea; (B) curve a, water; curve b, 8 M urea; curve c, 3 M ethylurea; curve d, 5 M ethylurea; curve e, 3 M propylurea; curve f, 5 M propylurea; (C) curve a, water; curve b, 8 M urea; curve c, 5 M ethylurea; curve d, 7 M methylurea; curve e, 1 M butylurea. All solutions in 0.1 M KCl, 0.02 M phosphate (pH 6.9) buffer. The protein concentrations used ranged from 0.004 to 0.073%.

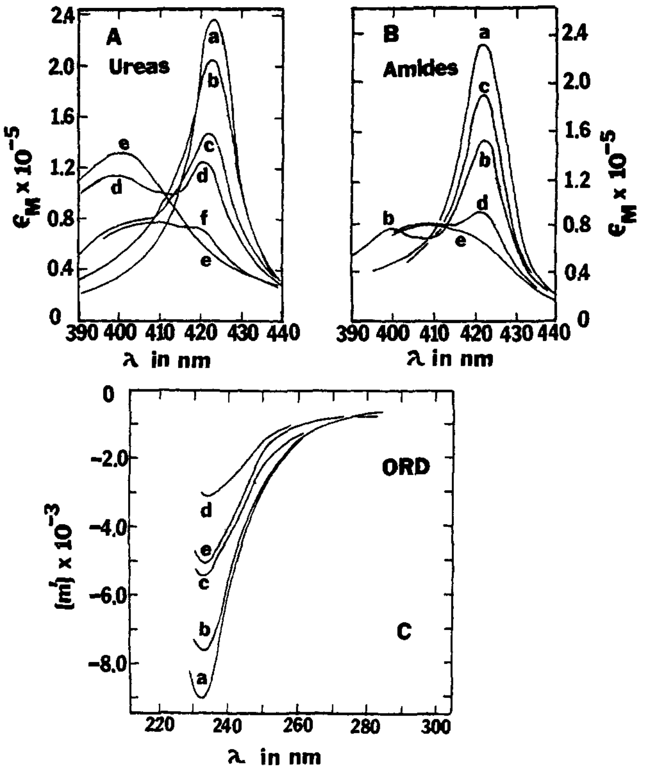

FIGURE 2: The denaturing effects of the ureas and amides on the absorbance in the Soret region and the ORD spectra of *Glycera dibranchiata* CO-hemoglobin (monomer): (A) curve a, water; curve b, 6 M urea; curve c, 6.4 M methylurea; curve d, 4.1 M ethylurea; curve e, 3 M propylurea; curve f, 1 M butylurea; (B) curve a, water; curve b, 10 M formamide; curve c, 7 M acetamide; curve d, 6 M propionamide; curve e, 3 M butyramide; (C) curve a, water; curve b, 6 M urea; curve c, 6.4 M methylurea; curve d, 5.6 M ethylurea; curve e, 1 M butylurea. All the solutions contained 0.1 M KCl–0.01 M phosphate (pH 7.0) buffer. The protein concentrations ranged from 0.0044 to 0.046%.

refractive index of the solvent at wavelength λ. The mean residue molecular weights, M_0, of 111 and 112.5 were used for the *Glycera* and human hemoglobins, respectively. The refractive index corrections given in the Handbook of Biochemistry were used. In the case of the ureas and amides for which refractive index data are not available, the aqueous $[m']_\lambda$ or $3/(n_\lambda{}^2 + 2)$ values were multiplied by the factor $(n_w{}^2 + 2)/(n_s{}^2 + 2)$ where n_w and n_s represent the refractive indices of water and the denaturing solvent used at the sodium D line.

For a given series of measurements volumetric dilutions were made using common stock solutions of protein and concentrated denaturing agent, in 5- or 10-ml stoppered volumetric flasks as previously described (Herskovits *et al.*, 1970a). Absorbance and ORD measurements were usually made after 0.5–2 hr equilibration at room temperature. Somewhat longer periods of equilibration of about 4 hr were required for denaturation with methylurea.

Results

Urea and Amide Denaturation Transitions. The denaturation behavior of both the four-chain human hemoglobin and the single-chain *Glycera dibranchiata* hemoglobin in various urea and amide solutions was investigated using both absorbance and optical rotatory dispersion (ORD) methods in following the denaturation transitions. Figures 1 and 2 show some of the spectrophotometric results in the visible Soret region and the spectropolarimetric results in the far-ultraviolet region obtained with some of the ureas and amides. The denaturation transitions as a function of urea and amide concentration obtained by these two methods are shown in Figures 3–5. Both techniques have given similar transition profiles, with essentially the same denaturation midpoints, S_m. As with the other globular proteins studied in our laboratory (Herskovits and Jaillet, 1969; Herskovits *et al.*, 1970a–c), the effectiveness of these two classes of solutes as protein denaturants is found to increase with increasing hydrocarbon content, requiring correspondingly lower concentrations to generate the characteristic inverted signmoidal shaped denaturation curves seen in these figures. Tables I and IV summarize the denaturation data in terms of the midpoints of the transitions, obtained with both hemoglobins, and the analysis of the data based on the Peller and Flory equations (Peller, 1959; Flory, 1957), the details of which will be examined in the Discussion.

The nature of the transitions can be analyzed in terms of native to unfolded protein equilibria (Kauzmann, 1959; Tanford, 1968; Sturtevant and Tsong, 1969; Cann, 1971; Brunori *et al.* 1972) with the order of the transition, *n*, defined by the empirical equation

$$K_{\text{app}} = C[\text{D}]^n \qquad (1)$$

where K_{app} is the apparent equilibrium constant, [D] is the denaturant concentration in moles per liter, and *C* is an empirical constant characteristic of each denaturant-protein system. The *n* value can be evaluated from log K_{app} *vs.* log

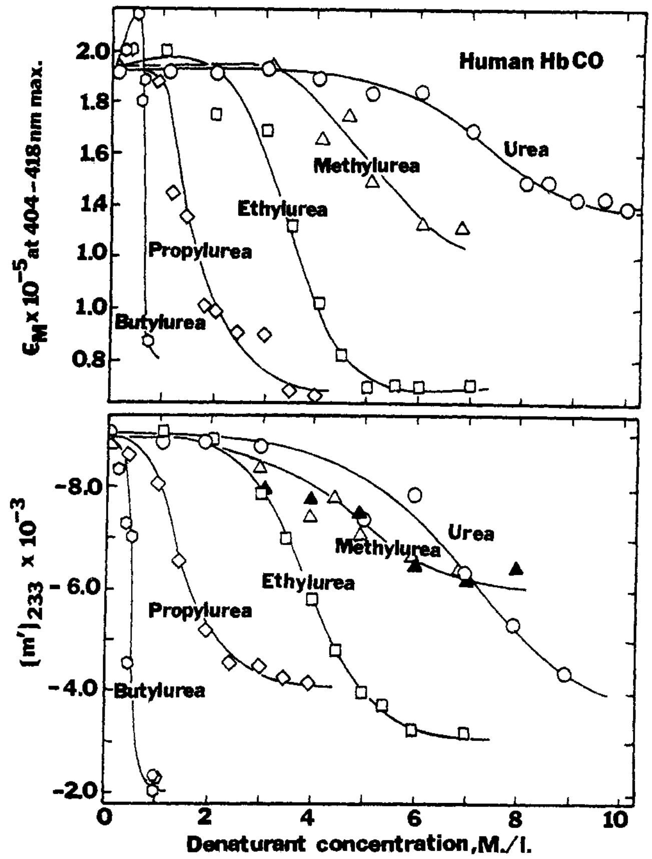

FIGURE 3: The denaturation of human CO-hemoglobin by various ureas at 25° followed by changes in absorbance at the 404–418-nm Soret band and ORD at 233 nm. Experimental conditions are as described in Figure 1.

[D] plots shown in Figure 6. Experimentally the K_{app} are calculated from the properly scaled ORD or absorbance curves (Sturtevant and Tsong, 1969) of Figures 3–5. In terms of the fraction of denatured protein f_D, $K_{app} = f_D/(1 - f_D)$. Table II presents the various n values for the ureas and amides with both human and *Glycera* hemoglobin. The range of n values is between about 4 and 6 for the ureas and somewhat higher for the amide series of denaturants. Surprisingly the order of the denaturation transitions n, that is an empirical measure of the sharpness of the transitions, seems to be unaffected by the number of hemoglobin chains in the particular hemoglobin investigated. The values for the tetrameric human hemoglobin are thus nearly the same as the n values of the monomer and polymer components of *Glycera* hemoglobin. On the other

TABLE I: Midpoints, S_m, of the Urea Denaturation Transition of Human Hemoglobin Based on Optical Rotatory Dispersion Measurements.[a]

	Ligand–Heme Oxidation State		
	O_2/Fe^{2+}	CO/Fe^{2+}	CN^-/Fe^{3+}
Urea	6.5	6.8	8.5
Methylurea	4.5	~5	7.0
Ethylurea	3.0	3.9	3.5
Propylurea	1.6	1.5	2.1
Butylurea	0.5	0.5	0.8
1,3-Dimethylurea		3.6	

[a] S_m values based on $[m']_{233}$ data of Figures 3 and 7.

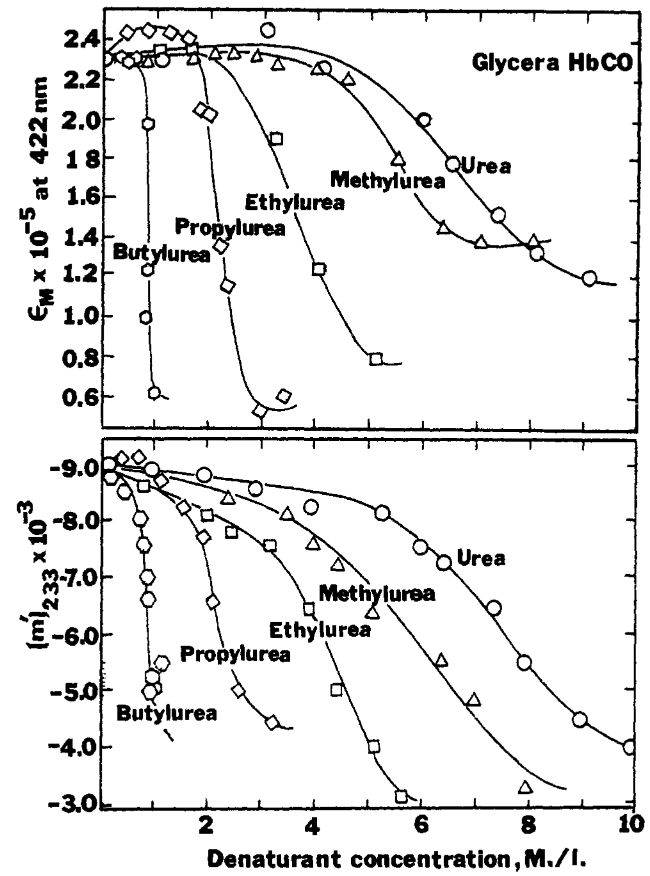

FIGURE 4: The denaturation of *Glycera dibranchiata* CO-hemoglobin (monomer) by various ureas at 25°. Denaturation followed by changes in absorbance at 422 nm and ORD at 233 nm. Experimental conditions given in Figure 2.

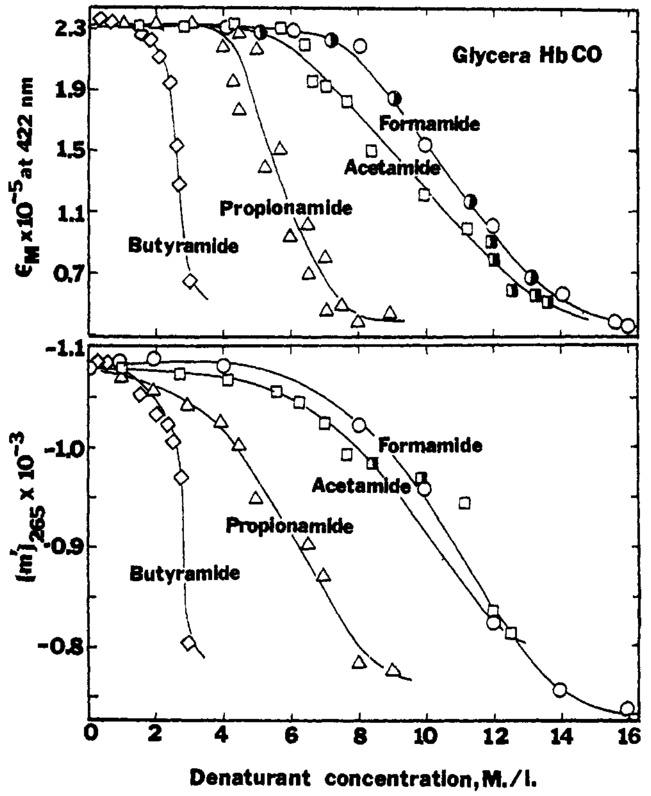

FIGURE 5: The denaturation of *Glycera dibranchiata* CO-hemoglobin (monomer) by the straight-chain amides at 25°. Denaturation followed by changes in absorbance at 422 nm and ORD at 265 nm. Experimental conditions are given in Figure 2.

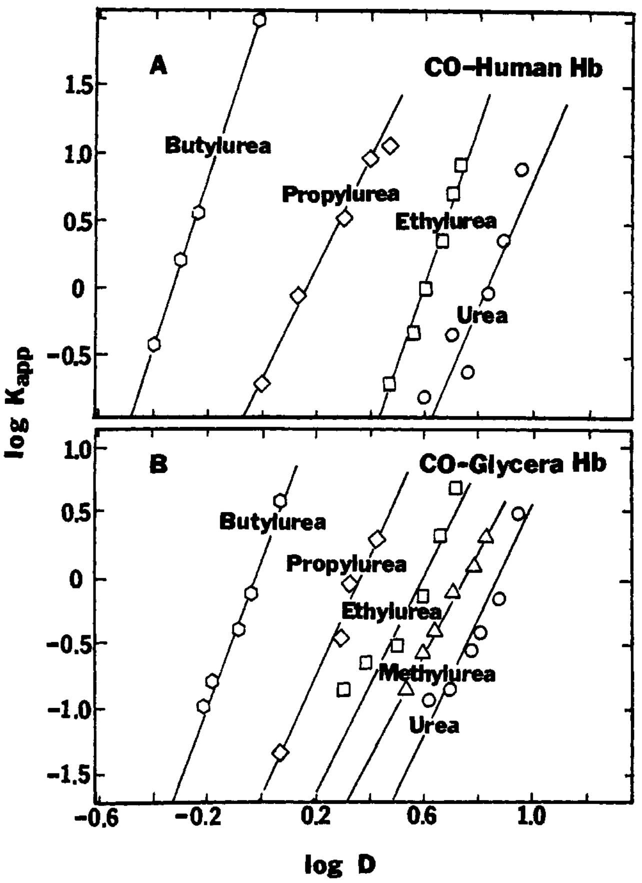

FIGURE 6: Plot of log K_{app} *vs.* logarithm of the concentration of various ureas, log [D]. The human and *Glycera* CO-hemoglobin plots are based on the ORD data of Figures 3 and 4. The *n* values based on the slopes for the different ureas are listed in Table II.

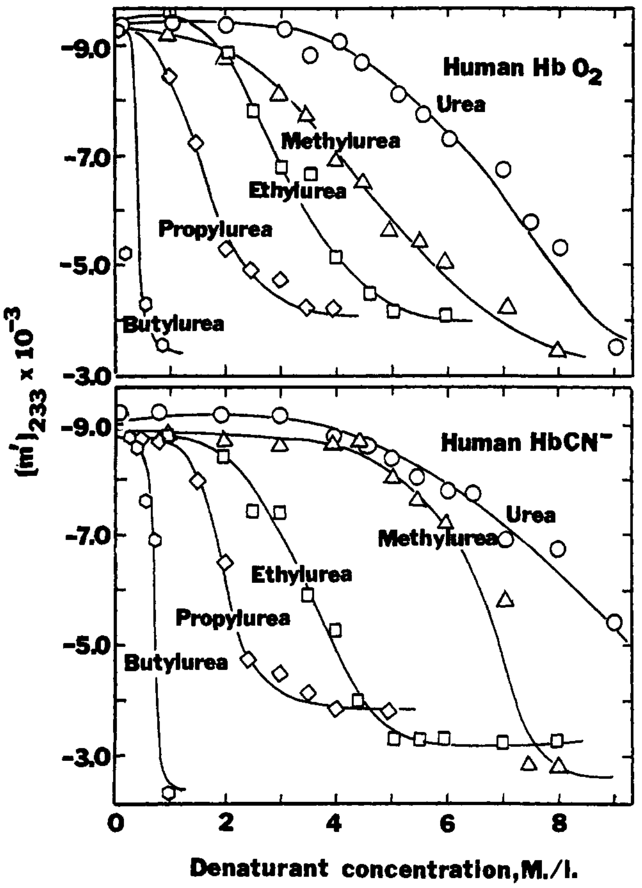

FIGURE 7: The denaturation of human oxyhemoglobin and cyanmethemoglobin by various ureas followed by changes in mean residue rotation, $[m']_\lambda$, at 233 nm. The protein concentrations ranged from 0.024 to 0.052%; all the solvents contained 0.1 M KCl–0.02 M phosphate (pH 6.9) buffer.

hand the values for sperm whale myoglobin are significantly different, more or less in the ranges of 8–13. It is interesting that Brunori *et al.* (1972) have obtained *n* values in the ranges of 10–13 for the alcohol denaturation of *Aplysia* myoglobin.

Effects of Ligands and Changes in Oxidation States. The changes in ligand binding are known to alter the stability of the tetrameric structure of human hemoglobin (Antonini, 1965; Guidotti, 1967). Guidotti (1967) found that the sequence of decreasing stability of the undissociated tetrameric form was deoxyhemoglobin > CO-hemoglobin > oxyhemoglobin ~ cyanmethemoglobin. In terms of urea denaturation, rather than subunit dissociation from the tetrameric form of $\alpha\beta$ dimers, our results show (Figures 3 and 7 and Table I) the same order of decreasing stability with the CO- and O_2-liganded ferrous heme states. There is, however, an inversion in changing to the ferric state. The cyanmet form of hemoglobin is found to be most stabilized and resistant to unfolding by the various ureas. Thus, for example, denaturation midpoints with urea, based on the ORD transi-

TABLE II: Values of the *n* Parameter in Eq 1 for Hemoglobins and Sperm Whale Myoglobin.[a]

	Glycera dibranchiata Hb		Human Hb			Sperm Whale Myoglobin
Solvent/Ligand	CO Monomer	CO Polymer[a]	O_2	CO	CN⁻	Fe^{3+}/H_2O
Urea	4.3	6.5	4.1	4.8	4.3	11.6
Methylurea	4.0	5.6	3.7		5.0	11.8
Ethylurea	4.1	6.1	4.9	6.4	5.0	12.6
Propylurea	4.5	5.2	3.8	4.3	4.9	7.8
Butylurea	5.5	3.0		6.0	5.0	6.2
Formamide	4.4			7.1		14
Acetamide	4.2			9.0		15
Propionamide	3.5			6.3		12
Butyramide	~4			12.4		10

[a] The *n* values given for the high molecular weight polymer component of *Glycera* hemoglobin (Seamonds *et al.*, 1971a) in the various ureas and the human CO-hemoglobin (CO-Hb) in the amides were based on absorbance measurements. The rest of the data is based on ORD measurements such as in Figures 3–5.

TABLE III: Effects of the Ureas on the Mean Residue Rotation, $[m']_\lambda$, at 233 nm and Helix Content of Carbonmonoxy Human and *Glycera dibranchiata* Hemoglobins.

Solvent	Human Hb		*Glycera* Hb	
	$[m']_{233}$	Helix Content[a]	$[m']_{233}$	Helix Content[a]
Water	−8800 ± 300	71	−8620 ± 300	69
9 M urea	−4440	26	−4510	27
8 M methylurea	−6420	47	−3280	14
7 M 1,3-dimethylurea	−3470	16	−4530	27
6 M ethylurea	−3520	16	−3100[b]	12
3 M propylurea	−4470	26	−4500[b]	27
1 M butylurea	−2220	3	−5250	34

[a] Calculated values based on eq 2, with the $[m']$ converted to $[m]_\lambda$ by dividing $[m']_\lambda$; by the dispersion factor, $3/(n^2 + 2)$, which is equal to 0.764 at 233 nm. [b] Data taken from smoothed curves of Figure 4.

tions of Figures 3 and 7, are found to be at 8.5, 6.8, and 6.5 M for CN⁻-, CO-, and O_2-liganded hemoglobins, respectively. The breadth of the transitions, on the other hand, appears to be largely unaffected by changes on the nature of the bound ligand and changes in the oxidation state of the heme groups. Essentially the same order or n values were obtained with O_2-hemoglobin and cyanomethemoglobin as with the CO-, with ranges in n from about 4 to 6 for the different urea transitions.

Conformation of the Denatured Hemoglobins. Table III presents a summary of the ORD data of human and the single-chain *Glycera* hemoglobin in various concentrated urea solutions. In addition to the mean residue rotation at 233 nm, $[m']_{233}$, we have also included estimates of the helix content remaining in these solvents, based on the recently published equations of Chen *et al.* (1972)

$$[m]_{233} = -12{,}700 f_H - 2520 \qquad (2)$$

where f_H represents the fraction of the polypeptide chain in the α-helical form. The reference parameters for $[m]_\lambda$ for the helical and unordered conformations in eq 2 are based on the known X-ray structure and ORD information in solution of five proteins. Our analysis of the urea denaturation data of this table on the two hemoglobins as well as our earlier findings with other proteins (Herskovits *et al.*, 1970c) indicate that the ureas as a class of denaturants stabilize the random coil form of proteins. Due to the strong absorption of the amides at 265 nm a similar analysis of the amide data could not be carried out. The less negative $[m']_{265}$ values of *Glycera* hemoglobin in the various amides above the denaturation transitions (shown in Figure 5) and the effects of these amides on the Moffitt–Yang b_0 parameters of myoglobin (Herskovits *et al.*, 1970b) indicate that this class of protein denaturants is also basically random coil promoting in character.

The absorbance profiles of hemoglobin solutions show no clearly defined isosbestic points (Figures 1 and 2) with some of the denaturants of this study. This suggests that the denatured states of hemoglobin are perhaps not the same, or that the known aggregation of the heme moiety upon denaturation (Polet and Steinhardt, 1969; Herskovits *et al.*, 1970b,c) complicates the interpretation of absorbance data. Kawahara *et al.* (1965) have investigated the effects of urea on the reversibility of CO-hemoglobin and have found that exposure of the protein to 6.4 or 8.0 M urea for short periods of time produced no irreversible changes in the sedimentation constants. Longer exposure of 2 or 5 days was found to cause slow changes in the properties of the protein, presumably due to aggregation. Factors controlling the reversibility of urea and amide denaturation of hemoglobin are presently being investigated in our laboratory.

Discussion

The denaturation of the single-chain *Glycera dibranchiata* and four-chain human hemoglobins by urea and amide series of denaturants is found to be closely similar to the denaturation behavior of the other globular single chain proteins, sperm whale myolgobin, cytochrome *c*, and α-chymotrypsinogen, due to these same two classes of solutes (Herskovits and Jaillet, 1969; Herskovits *et al.*, 1970b,c). Despite the moderate differences in the order or steepenss in the denaturation transitions found for different proteins such as the hemoglobins and sperm whale myoglobin (Table II), the midpoints of the transitions are found to be remarkably similar, exhibiting the same order of increasing effectiveness of the denaturant with increasing chain length of the alkyl portions of the series of increasing hydrocarbon content.

It is possible to examine and predict the midpoints of the transitions from native to denatured or unfolded protein forms by means of the equations of Peller (1959) and Flory (1957) that have been employed with varying success to account for the effects of salts (Mandelkern and Steward, 1964; Robinson and Grant, 1966; Schrier and Schrier, 1967; Von Hippel and Schleich, 1969) and water miscible neutral solutes of the alcohol (Schrier *et al.*, 1965; Herskovits *et al.*, 1970a; Herskovits and Harrington, 1972; Parodi *et al.*, 1973), urea (Herskovits *et al.*, 1970c), and amide (Herskovits *et al.*, 1970b) classes on the stability of proteins and other biopolymers. The lowering of the melting temperature or denaturation midpoint of a biopolymer, ΔT_m, due to preferential interaction with the denaturing solute of activity, a_D, per average monomer unit, in the unfolded (*i.e.*, denatured) form is given by the equation first derived by Peller (1959)

$$\Delta T_m = T_m^0 - T_m = (RT_mT_m^0/\Delta h)\bar{v}\ln(1 + K_Ba_D) \qquad (3)$$

where T_m and T_m^0 are the midpoints of the denaturation transition temperature in the presence and absence of denaturant, Δh is the enthalpy change of unfolding per monomer unit, $\bar{v}$ is the effective number or fraction of binding sites per monomer unit, and K_B is the association or binding constant of the denaturant with the average monomer unit. For relatively weak interactions, with K_Ba_D much smaller than one, expansion of the logarithmic term of eq 3, and the activity coefficient

TABLE IV: A Comparison of the Experimental and Calculated Denaturation Midpoints for Human and *Glycera dibranchiata* Carboxyhemoglobins and Sperm Whale Myoglobin at 25°.

	Human Hemoglobin		Glycera Hemoglobin		Myoglobin[a]	Calculated[b]		
	Absorbance (404–418 nm)	ORD (233 nm)	Absorbance (422 nm)	ORD (233 nm)	Absorbance (409 nm)	Eq 5 with K_S Data of a Tryp E $\bar{\nu} = 0.1$	Eq 4 with $K_{H\Phi}$ from ΔF_{tr} Data Alone $\bar{\nu} = 1.0$	Eq 4 with K_B ($K_{H\Phi} + K_P$) $\bar{\nu} = 0.6$
				Ureas				
Urea	7.3	6.8	6.6	7.2	6.6	6.3		7.1
Methylurea	5.0	~5	5.4	5.6	5.6	3.7	13.4	5.4
Ethylurea	3.5	3.9	3.6	4.1	3.8	2.3	4.7	3.7
Propylurea	1.6	1.5	2.1	2.1	1.8	1.6	1.8	2.1
Butylurea	0.6	0.5	0.9	0.9	0.6	1.1	0.7	0.98
1,3-Dimethylurea	3.5	3.6	5.6	4.8	5.4	2.0	6.7	4.3
				Amides		$\bar{\nu} = 0.06$		
Formamide	12.9		10.6	10.5	9.4	9.1		10.8
Acetamide	9.5		9.6	10.0	7.7	6.7	13.4	7.3
Propionamide	4.8		5.5	5.7	4.4	4.6	4.7	4.6
Butyramide	2.2		2.6	2.7	2.3	3.2	1.8	2.3
Dimethylformamide	5.0		5.0		7.8	3.1	6.7	5.5
Dimethylacetamide	4.4		4.5		7.5	1.9	4.5	4.4

[a] Data of Herskovits *et al.* (1970b,c). [b] Parameters employed with eq 4 and 5: $T_m = 298°$, $T_m^0 = 355$°K, and $\Delta h = 500$ cal/mol of peptide. For K_B calculations with eq 4: $K_{H\Phi} = 1.01, 2.88, 7.59$, and 20×10^{-2} for each methyl, ethyl, propyl, or butyl group contribution, and $K_P = 3.2$ and 2.1×10^{-2} for the polar $NH(CO)NH_2$ and $CONH_2$ urea and amide group contributions. The $K_{H\Phi}$ are based on free-energy transfer data (Herskovits *et al.*, 1970a) while the K_P values are calculated using the average denaturation midpoints obtained with urea and formamide of this table and Table I. The K_S values used with eq 5 were based on solubility data of *N*-acetyl-L-tryptophan ethyl ester data in the various ureas and amides listed (Herskovits *et al.*, 1970b,c).

γ_D, taken as one, the solvent denaturation midpoint, S_m, can be expressed as

$$S_m = c_D\gamma_D = (\Delta T_m \Delta h/RT_mT_m^0\bar{\nu})1/K_B \qquad (4)$$

Similar expressions have been obtained, using the Setschenow constants, K_S (Long and McDevit, 1952), based on solubilities of the appropriate model compounds to represent the average monomer unit of the biopolymer (Schrier and Schrier, 1967; Herskovits *et al.*, 1970a–c; Herskovits and Harrington, 1972), with the denaturation midpoint being equal to

$$S_m = (-\Delta T_m \Delta h/2.303RT_mT_m^0\bar{\nu})1/K_S \qquad (5)$$

In any discussion of specific *vs.* nonspecific binding effects (Tanford, 1968; Von Hippel and Schleich, 1969; St. Pierre and Jencks, 1969; Von Hippel *et al.*, 1973), through the binding constant, K_B, in eq 4, emphasis inadvertantly centers on the specificity or physical binding of the denaturant or salt to specific sites on the biopolymer. With the use of eq 5, through the equivalent Setschenow constant, K_S, the emphasis is now on the nonspecific solubilizing aspect of the denaturant or salt. Actually, in the approach adopted in our studies (Herskovits *et al.*, 1970a–c) both elements of nonspecific solvent effects and specific "binding" effects of the denaturant to the biopolymer are utilized for the evaluation of K_B. The hydrophobic component of K_B is evaluated by use of standard free energy of binding expression

$$\Delta F°_B = -RT \ln K_B = (\Delta F°_{H\Phi} - T\Delta S_B°) \qquad (6)$$

with $\Delta F°_{H\Phi}$ based either on free-energy transfer or solubility data taken from the literature (Cohn and Edsall, 1943; Tanford, 1962; Brandts, 1964) or the Scheraga–Nemethy theory of hydrophobic bonding (Nemethy and Scheraga, 1962; Schrier *et al.*, 1964, 1965). In this relation it should be noted that $\Delta S°_B$ represents the loss of translational entropy on binding of the denaturant to the biopolymer, estimated from the dimerization of model compounds in water (Schrier *et al.*, 1965).

The studies of Schrier and Schrier (1967) have demonstrated that in a series of related solutes the Setschenow constants K_S may be approximated by assuming that the K_S values of the constituent nonpolar and polar groups are additive. Additivity of group contributions to K_B has been used in our studies dealing with the denaturing effects of the amides on sperm whale myoglobin, cytochrome *c*, and α-chymotrypsinogen (Herskovits *et al.*, 1970b). In terms of the constituent nonpolar or hydrophobic $K_{H\Phi}$ and polar K_P components, the binding constant K_B to be used in conjunction with eq 4 may be expressed as

$$K_B = K_{H\Phi} + K_P \qquad (7)$$

where K_P represents the $NH(CO)NH_2$ and the $CONH_2$ contributions to the overall urea or amide–peptide group interaction. T_m^0 and Δh values of 355°K and 500 cal/mol per average peptide group (Hermans and Acampora, 1967) were used for our S_m calculations for myoglobin (Herskovits *et al.*, 1970a–c) resulting in a value of 0.136 for the constant $(\Delta T_m \Delta h/RT_m^0T_m)$ in eq 4, at 25°. Since the denaturation midpoints of the single- and four-chain *Glycera* and human hemoglobins with the various ureas and amides are found to be largely the same as those observed on sperm whale myoglobin (Table IV) the same constant was used to calculate the S_m values of the two hemoglobins of this study.

Table IV presents a comparison of the denaturation midpoints, S_m, of these two hemoglobins and myoglobin, based on absorbance measurements in the Soret region and ORD measurements (Figures 3–5) with the calculated S_m parameters obtained by means of eq 4, 5, and 7. The calculations based on both eq 4 and 5 give relatively satisfactory agreement with the experimental results, with the Setschenow constants, K_S, obtained from solubility data of the *N*-acetyl ethyl ester of tryptophan in urea and amide solutions (Herskovits *et al.*, 1970b,c) and the binding constants, K_B, evaluated by use of eq 7. With somewhat less success, the amide data of sperm whale myoglobin have also been fitted (Herskovits *et al.*, 1970b), using $K_{H\Phi}$ values based on the Scheraga–Nemethy theory of hydrophobic bonding. The most significant conclusion based on the calculations of this table is that the nonpolar, hydrophobic contributions of the increasingly larger alkyl chains of the higher members of these two series of denaturants play an increasingly more dominant role in the denaturation process, by stabilizing the unfolded form of protein chains through hydrophobic interactions. This is clearly demonstrated by the trend of the calculated S_m values based on the nonpolar, alkyl chain contributions to the binding constants, $K_{H\Phi}$ alone, given in the central column (7) of Table IV. In contrast to the behavior of the alcohol series of denaturants (Schrier *et al.*, 1965; Herskovits *et al.*, 1970a) it is not possible to ignore the polar amide group contributions to the binding constants in the case of the lower members of the urea and amide series. This is not to say that hydrophobic effects are absent or have little to do with the denaturation in these solvents; the solubilizing effects of urea and formamide based on model and detergent studies (Nozaki and Tanford, 1963; Wetlaufer *et al.*, 1964; Herskovits *et al.*, 1970b) have been interpreted as being at least partly due to hydrophobic interactions and destabilization of micellar structures (Bruning and Holtzer, 1961; Gratzer and Beaven, 1970).

The results of the osmometric and light scattering studies of the accompanying paper (Elbaum and Herskovits, 1974) suggest that the rank or order of effectiveness of the ureas and amides is the same as both subunit dissociating agents and protein denaturants. It should therefore be possible to use the microscopic binding constants, K_B, derived in the present work to account for the observed effects of hydrophobic solutes on the subunit organization of proteins.

References

Antonini, E. (1965), *Physiol. Rev. 45*, 123.

Brandts, J. F. (1964), *J. Amer. Chem. Soc. 86*, 4302.

Bruning, W., and Holtzer, A. (1961), *J. Amer. Chem. Soc. 83*, 4865.

Brunori, M., Giacometti, G. M., Antonini, E., and Wyman, J. (1972), *J. Mol. Biol. 63*, 139.

Cann, J. R. (1971), *Biochemistry 10*, 3707.

Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972), *Biochemistry 11*, 4120.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, Chapter 9.

Elbaum, D., and Herskovits, T. T. (1974), *Biochemistry 13*, 1268.

Elbaum, D., Pandolfelli, E. R., and Herskovits, T. T. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32*, 571.

Flory, P. J. (1957), *J. Cell. Comp. Physiol. 49*, 175.

Gratzer, W. B., and Beaven, G. H. (1970), *J. Phys. Chem. 73*, 2270.

Guidotti, G. (1967), *J. Biol. Chem. 242*, 3685.

Hermans, J., Jr., and Acampora, G. (1967), *J. Amer. Chem. Soc. 89*, 1547.

Herskovits, T. T., Gadegbeku, B., and Jaillet, H. (1970a), *J. Biol. Chem. 245*, 2588.

Herksovits, T. T., and Harrington, J. P. (1972), *Biochemistry 11*, 4800.

Herskovits, T. T., and Jaillet, H. (1969), *Science 163*, 282.

Herskovits, T. T., Jaillet, H., and DeSaena, A. (1970b), *J. Biol. Chem. 245*, 6511.

Herskovits, T. T., Jaillet, H., and Gadegbeku, B. (1970c), *J. Biol. Chem. 245*, 4544.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Kawahara, K., Kirshner, A. G., and Tanford, C. (1965), *Biochemistry 4*, 1203.

Long, F. A., and McDevit, W. G. (1952), *Chem. Rev. 51*, 119.

Mandelkern, L., and Steward, W. E. (1964), *Biochemistry 3*, 1135.

Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.

Nozaki, Y., and Tanford, C. (1963), *J. Biol. Chem. 238*, 4074.

Parodi, R. M., Bianchi, E., and Ciferri, A. (1973), *J. Biol. Chem. 248*, 4047.

Peller, L. (1959), *J. Phys. Chem. 63*, 1199.

Polet, H., and Steinhardt, J. (1969), *Biochemistry 8*, 587.

Robinson, D. R., and Grant, M. E. (1966), *J. Biol. Chem. 241*, 4030.

Schrier, E. E., Ingwall, R. T., and Scheraga, H. A. (1965), *J. Phys. Chem. 69*, 298.

Schrier, E. E., Pottle, M., and Scheraga, H. A. (1964), *J. Amer. Chem. Soc. 86*, 3444.

Schrier, E. E., and Schrier, E. B. (1967), *J. Phys. Chem. 71*, 1851.

Seamonds, B., Forster, R. E., and George, P. (1971a), *J. Biol. Chem. 246*, 5391.

Seamonds, B., Forster, R. E., and Gottlieb, A. J. (1971b), *J. Biol. Chem. 246*, 1700.

St. Pierre, T., and Jencks, W. P. (1969), *Arch. Biochem. Biophys. 133*, 99.

Sturtevant, J. M., and Tsong, T. Y. (1969), *J. Biol. Chem. 244*, 4942.

Tanford, C. (1962), *J. Amer. Chem. Soc. 84*, 4240.

Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.

Von Hippel, P. H., Peticolas, V., Schack, L., and Karlson, L. (1973), *Biochemistry 12*, 1256.

Von Hippel, P. H., and Schleich, T. (1969), *in* Structure and Stability of Biological Macromolecules, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 417.

Wetlaufer, D. B., Malik, S. K., Stoller, L., and Coffin, L. R. (1964), *J. Amer. Chem. Soc. 86*, 508.