Noncovalent Inhibitors of Sickle Hemoglobin Gelation: Effects of Aliphatic Alcohols, Amides, and Ureas†

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ABSTRACT: The effects of three classes of organic cosolvents (aliphatic alcohols, amides, and ureas) on the gelation of deoxygenated sickle hemoglobin (deoxy-Hb S) were evaluated. The equilibrium solubility was measured as the saturation concentration, c_{sat}, after phase separation by centrifugation at 30 °C. For the members of each class, c_{sat} increased linearly with concentration, indicative of an inhibition of polymerization. Within each class, these solubilizing effects were proportional to the hydrocarbon content of the alkyl moiety, expressed as the number of effective methylene units, n. Among the three classes of perturbants, for the same n value, the effectiveness in increasing solubility varied according to the order ureas > amides > alcohols. Moreover, the Setschenow constant, K_s, a quantitative measure of molar effectiveness derived from the solubility data, could be separated into two components, both hydrophobic in nature: one arising from the functional group and the other from the pendant aliphatic chain. In addition, the functional group modulated the expression of the hydrophobic character of the alkyl group in the order $NHCONH_2 > CONH_2 > OH$. These results, considered in conjunction with our studies of lyotropic salts [Poillon, W. N., & Bertles, J. F. (1979) *J. Biol. Chem. 254*, 3462–3467], strongly suggest that electrostatic forces and hydrogen bonding play only a subsidiary role in stabilizing the polymer. For the most part, hydrophobic interactions, especially the one specified by the substitution β6(A3)Glu → Val, are the primary determinant of the stability of the deoxy-Hb S polymer. These findings also suggest that K_s values of other types of noncovalent perturbants might be expressed as an additive function of their constituent groups. A tabulation of such K_s values would then provide a means by which the components of any potential antisickling agent could be varied so as to maximize its ability to inhibit gelation.

The underlying basis of the sickling phenomenon is the intracellular polymerization of sickle hemoglobin (Hb S)[1] which occurs upon deoxygenation of erythrocytes from patients homozygous for Hb S (SS erythrocytes) (Harris, 1950; Bertles & Döbler, 1969). The development of a therapeutically effective antisickling agent depends upon a precise understanding of the nature of the noncovalent interactions which stabilize the supramolecular structure of the deoxy-Hb S polymer. The negative temperature coefficient of gelation exhibited by concentrated solutions of deoxy-Hb S indicates that the predominant stabilizing force is hydrophobic in nature (Murayama, 1957). Nevertheless, electrostatic and hydrogen-bonding interactions could contribute to the stabilization energy of the polymer as well (Bookchin & Nagel, 1973; Briehl & Ewert, 1973; Freedman et al., 1973).

The systematic evaluation of the effects of lyotropic salts on the equilibrium solubility, c_{sat}, of deoxy-Hb S was described in an earlier paper (Poillon & Bertles, 1979). The results showed that each salt exerted either a stabilizing (c_{sat} decreased) or a destabilizing (c_{sat} increased) effect, depending on the specific cation and anion involved. Such specificity precluded electrostatic shielding as being responsible for the effects observed. Rather, the differential response of the solubility to individual cations and anions could be explained more reasonably as arising from the solvent-mediated perturbation of those hydrophobic forces which stabilize the polymer. The present study was undertaken in order to evaluate the effects of three classes of organic cosolvents (aliphatic alcohols, amides, and ureas) on the solubility of deoxy-Hb S, as well as to assess the extent to which each of the three fundamentally distinct kinds of noncovalent interactions contributes to the overall stability of the Hb S fiber.

Experimental Procedures

Sickle Hemoglobin. Venous blood, anticoagulated with EDTA, was obtained over a 1-year period from the same patient whose blood was used in our previous study (Poillon & Bertles, 1979). The procedure for preparation of concentrated hemolysates (32–36 g/dL) from SS erythrocytes has already been described (Poillon & Bertles, 1979). Because such hemolysates contained <2% Hb F, they could be used directly, without further purification, after extensive dialysis against 0.05 M Bis-Tris buffer (pH 6.80 at 30 °C) in the cold.

Organic Additives. The best quality reagent grade additives were obtained from various commercial sources and used without further purification. Piracetam was provided by Dr. R. H. Chesky of the Warner-Lambert/Parke-Davis Co., Ann Arbor, MI. Any of the aliphatic amides or ureas which were obviously deliquescent were dried to constant weight in vacuo over P_2O_5. Concentrated stock solutions (usually 2–4 M) were prepared in distilled water. Because the final concentration of organic cosolvent was <1 M in most cases, its effect on the dielectric constant of the solvent was negligible.

Solubility Measurements. The method for determining the equilibrium solubility of deoxy-Hb S by ultracentrifugation, as well as its modification to a microscale, has been described elsewhere (Magdoff-Fairchild et al., 1976; Poillon & Bertles, 1979). Samples of 400 μL total volume were centrifuged for 75 min at 242000*g* in an SW 50.1 rotor at 30 °C. After centrifugation the pH of the supernatant phase was measured

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[1] Abbreviations used: Hb S, sickle hemoglobin; c_{sat}, saturation concentration; MGC, minimum gelling concentration; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; SS erythrocytes, erythrocytes from patients homozygous for Hb S; Hb F, fetal hemoglobin; Hb A, adult hemoglobin; Piracetam, the cyclic lactam derivative of γ-aminobutyric acid and acetamide.

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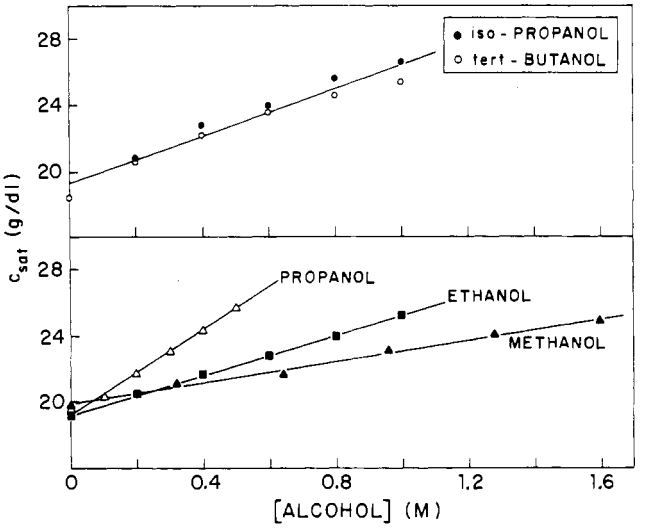

FIGURE 1: Effects of aliphatic alcohols on the equilibrium solubility, c_{sat}, of deoxy-Hb S. In all cases, Hb S samples were in 0.05 M Bis-Tris buffer, pH 6.80 at 30 °C. After deoxygenation, samples were equilibrated for 1 h and phase separation was achieved by centrifugation at 242000*g* for 75 min at 30 °C. The concentration of Hb S in the supernatant phase corresponds to c_{sat}.

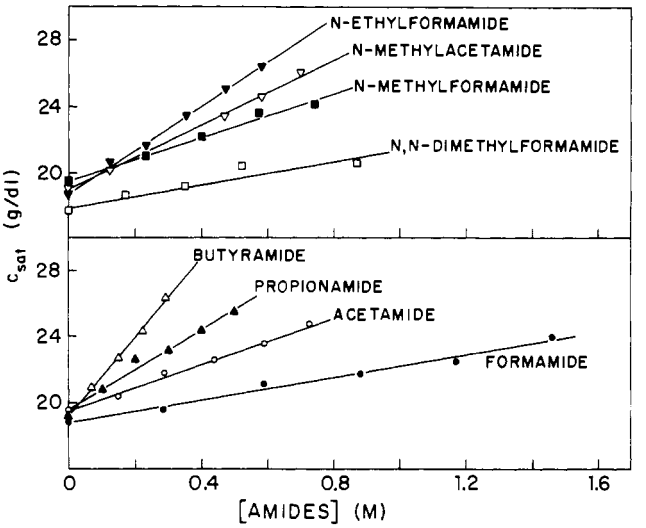

FIGURE 2: Effects of aliphatic amides on the equilibrium solubility, c_{sat}, of deoxy-Hb S. Other experimental conditions are given in the legend to Figure 1.

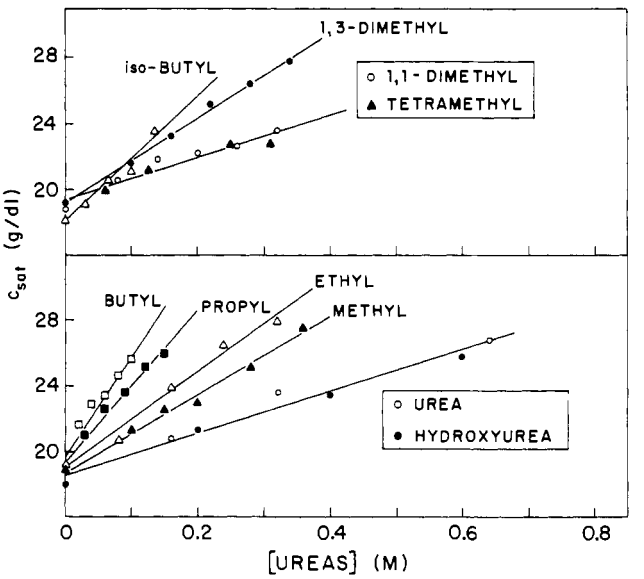

FIGURE 3: Effects of alkylureas on the equilibrium solubility, c_{sat}, of deoxy-Hb S. Other experimental conditions are given in the legend to Figure 1.

(Magdoff-Fairchild et al., 1976). In no case did the measured pH deviate by more than ±0.20 from the nominal value of 6.80. Conversion of oxy- to deoxy-Hb S was achieved with a twofold molar excess of sodium dithionite; the initial concentration of Hb S was in the range of 25–29 g/dL. Because of variable dilution incurred by the addition of different organic cosolvents, the final concentration of Bis-Tris buffer ranged from 36 to 45 mM.

Results

Base-Line Solubility. Overall, the effects of 26 organic additives on the equilibrium solubility, c_{sat}, of deoxy-Hb S were evaluated. In each experiment, a control sample (in the absence of additive) was included. This solubility corresponds to that in 0.05 M Bis-Tris buffer and is considered equivalent to that which would pertain in water. Statistical analysis of the solubility data accumulated for such controls ($n = 33$) gave a mean value of $c_{sat}° = 19.0 \pm 0.7$ g/dL. The standard deviation indicated corresponds approximately to the experimental error (±4%) inherent in such solubility measurements.

Aliphatic Alcohols. Solubility profiles (c_{sat} vs. concentration) for the first three homologues of the straight-chain aliphatic alcohol series are shown in the lower half of Figure 1. The effectiveness in increasing the solubility of deoxy-Hb S varied according to the order propanol > ethanol > methanol. In the upper half of Figure 1 are shown solubility profiles for two branched-chain aliphatic alcohols, 2-propanol and 2-methyl-2-propanol. Because both additives increased the solubility to the same extent, the data for each are plotted on the same line.

Aliphatic Amides. The solubility of deoxy-Hb S as a function of the concentration of the first four homologues of the straight-chain aliphatic amide series is shown in the lower half of Figure 2. The order of effectiveness was butyramide > propionamide > acetamide > formamide. In the upper half of Figure 2 are shown solubility profiles for three monoalkyl-substituted amides and one dialkyl-substituted amide. The order of effectiveness was *N*-ethylformamide > *N*-methylacetamide ≫ *N*,*N*-dimethylformamide. Moreover, while *N*-methylformamide was considerably less effective than *N*-ethylformamide, it was essentially equivalent in effectiveness to acetamide, its isomeric analogue (C_2H_5NO). Furthermore, Piracetam, a cyclic acetamide derivative reputed to have antisickling activity, was only slightly more effective than its parent compound acetamide.

Alkylureas. The effects of the first four homologues of the straight-chain monoalkyl-substituted ureas, as well as unsubstituted urea and hydroxyurea, on the solubility of deoxy-Hb S are shown in the lower half of Figure 3. The order of effectiveness was butylurea > propylurea > ethylurea > methylurea > urea ~ hydroxyurea. In the upper half of Figure 3 are shown the solubility profiles for assorted mono-, di-, and tetra-substituted ureas. The order of effectiveness here was isobutylurea > 1,3-dimethylurea > 1,1-dimethylurea ~ tetramethylurea. It is noteworthy that for the dimethylureas, the 1,1-substituted derivative was considerably less effective than the 1,3-substituted one. Furthermore, tetramethylurea was only as effective as 1,1-dimethylurea, while 1,3-dimethylurea was nearly as effective as ethylurea. The slopes of the various solubility plots for the three classes of organic cosolvents depicted in Figures 1–3 have been calculated by linear regression analyses of the data and are compiled in Table I.

Thermodynamic Analysis of Solubility Data. The polymerization of deoxy-Hb S under conditions of thermodynamic equilibrium may be depicted as

$$n\text{Hb} \rightleftharpoons (\text{Hb})_n$$

The perturbation of this two-phase equilibrium by the three classes of organic additives examined is reflected in all cases by an elevation in solubility, indicative of an inhibition of polymerization. Since the polymerization is nucleation controlled (Williams, 1973; Hofrichter et al., 1974; Behe & Englander, 1978), its equilibrium constant corresponds to $1/c_{sat}$, while that for depolymerization, the direction appropriate for solubility data, corresponds to c_{sat}. From the mean value of the base-line solubility given earlier, the free energy change associated with depolymerization in the absence of perturbant

$$\Delta G_{D,w} = -RT \ln c_{sat}^{\circ} \quad (1)$$

was calculated to be 3.51 ± 0.03 kcal/mol. The nonideal behavior which prevails in such concentrated deoxy-Hb S solutions (Ross & Minton, 1977; Ross et al., 1978) as those used here makes it necessary to use activity, rather than concentration, to calculate $\Delta G_{D,w}$ (eq 1). By use of the tabulated values given for the concentration dependence of $\ln \gamma_2$ by Ross & Minton (1977), the corrected value of 4.16 ± 0.07 kcal/mol was obtained for this parameter.

The additional free energy change associated with depolymerization at 30 °C in the presence of any particular organic perturbant is given by

$$\Delta G_{perturbant} = (2.303)RTK_s c_s \quad (2)$$

where c_s is the molar concentration of perturbant and K_s is the Setschenow constant, the magnitude of which is a quantitative measure of the molar effectiveness of a given additive in elevating solubility.[2] Values of K_s were obtained from the solubility data of Figures 1–3, as described in the legend to Table I. Values of K_s and $\Delta G_{perturbant}$ for each of the organic cosolvents studied are compiled in Table I. The overall free energy change associated with depolymerization for any particular perturbant ($\Delta G_{D,perturbant}$) is then given by the sums of eq 1 and 2.[3] Values of $\Delta G_{D,perturbant}$ calculated by this relation range from 4.14 kcal/mol for methanol, the least effective perturbant, to 3.84 kcal/mol for butylurea, the most effective perturbant of the polymer ⇌ monomer equilibrium.

Discussion

A tabulation of the various solubility and thermodynamic parameters for the three classes of organic cosolvents is given in Table I. These data show that there are two distinct kinds of hydrophobic contributions to the overall solubilizing effects observed, one arising from the pendant alkyl group and one from the functional group to which the alkyl moiety is attached. In order to appreciate this distinction more easily, we will evaluate the data of Table I systematically, first by intraclass and then by interclass comparisons. This approach permits one to discriminate readily between the role of the alkyl moiety (intraclass) and the role of the functional group (interclass). The concept of effective methylene ($-CH_2-$) units, n, per alkyl chain will be used to facilitate these comparisons (Schrier & Scheraga, 1964; Schrier et al., 1965; von Hippel & Wong, 1965).[4] Since the effectiveness of any organic

Table I: Parameters Showing the Relationship between Solubility of Deoxy-Hb S and Concentration of Various Hydrophobic Perturbants

perturbant	carbon series	slope [g (dL M)$^{-1}$][a]	K_s (M^{-1})[b]	$\Delta G_{perturbant}$ (cal/mol)[c]	n[d]
		Alcohols and Glycols			
methanol	C_1	3.2	−0.06	−17	1
ethanol	C_2	5.8	−0.12	−33	2
propanol	C_3	12.7	−0.25	−70	3
2-propanol	C_3	7.1	−0.14	−39	2
2-methyl-2-propanol	C_4	7.1	−0.14	−39	2
ethylene glycol	C_2	2.5	−0.05	−14	
glycerol	C_3	3.5	−0.07	−20	
		Amides			
formamide	C_1	3.5	−0.07	−20	0
acetamide	C_2	7.1	−0.14	−39	1
propionamide	C_3	11.8	−0.23	−64	2
butyramide	C_4	23.5	−0.45	−125	3
N-methyl-formamide	C_2	6.7	−0.13	−36	1
N-ethyl-formamide	C_3	13.4	−0.26	−72	2
N-methyl-acetamide	C_3	9.8	−0.19	−53	(2)[e]
N,*N*-dimethyl-formamide	C_3	3.6	−0.08	−22	
Piracetam	C_6	9.7	−0.19	−53	
		Ureas			
urea	C_1	12.8	−0.25	−70	0
hydroxyurea	C_1	12.8	−0.25	−70	0
methylurea	C_2	23.9	−0.45	−125	1
ethylurea	C_3	29.3	−0.54	−150	2
propylurea	C_4	45.9	−0.89	−247	3
butylurea	C_5	60.3	−1.16	−323	4
isobutylurea	C_5	37.8	−0.80	−222	3
1,3-dimethyl-urea	C_3	25.6	−0.48	−133	(2)[e]
1,1-dimethyl-urea	C_3	12.7	−0.26	−72	
tetramethyl-urea	C_5	12.7	−0.26	−72	

[a] Evaluated by linear regression analyses of the solubility data presented in Figures 1–3; correlation coefficients, r values, were ≥0.98 in all cases. [b] Setschenow constants, K_s values, were obtained from the relation $\log S_0/S = K_s c_s$, using the solubility data of Figures 1–3 to evaluate the ratio S_0/S, where S_0 and S represent the respective molar solubilities of deoxy-Hb S in the absence or presence of perturbant at any given concentration, c_s. The magnitude of K_s is directly proportional to molar effectiveness. [c] Determined from the relation $\Delta G_{perturbant} = (2.303)RTK_s c_s$, at $c_s = 0.2$ M and 30 °C. This parameter reflects the extent to which the polymer ⇌ monomer equilibrium is perturbed by any given organic additive. [d] Effective methylene units; defined relative to the straight-chain homologues for each perturbant class. Branched-chain species are considered only as effective as the straight-chain homologue equivalent in length to the unbranched portion of the alkyl moiety. [e] Provisional values of $n = 2$ have been assigned to these two species because each is nearly as effective as its isomeric analogue containing an ethyl group. However, because either an amide or urea functionality is interposed between the two methyl groups, the value of n does not conform to its strict definition.

[2] It should be noted that because the solubility profile for any given perturbant (Figures 1–3) represents a constant activity isotherm, the ratio S_0/S used to evaluate the Setschenow constant corresponds to the relative activity coefficient ($\gamma/\gamma_0 = \gamma_{rel}$) and the values of $\Delta G_{perturbant}$ obtained with this relation are thermodynamically correct without further consideration of nonideality.

[3] The complete derivation of this thermodyamic analysis may be found in the miniprint supplement to our earlier paper (Poillon & Bertles, 1979) which dealt with the effects of lyotropic salts on the solubility of deoxy-Hb S.

[4] According to this theory, the effectiveness of an organic cosolvent in perturbing the hydrophobic forces which stabilize the native state of a globular protein is determined by the hydrocarbon content of the pendant alkyl moiety. For an unbranched aliphatic side chain, each carbon atom contributes one effective methylene unit; for branched-chain alkyl groups, the value of n corresponds to the number of carbon atoms equivalent in length to the unbranched portion of the chain. Unless noted otherwise, values of n given in Table I have been assigned on this basis.

cosolvent in perturbing the forces which stabilize the deoxy-Hb S polymer is reflected by the magnitude of its Setschenow constant (see Table I), all subsequent comparisons will be made on the basis of K_s values.

The effectiveness of straight-chain alcohols in increasing solubility was proportional to the length of the aliphatic side chain. Branched-chain alcohols also conformed to the concept of effective methylene units. Thus, 2-propanol and 2-methyl-2-propanol were essentially equipotent with ethanol, and each has an n value of 2. Furthermore, both ethylene glycol and glycerol (solubility profiles not shown) were about as effective as methanol. It seems, therefore, that for the C_2 and C_3 series the presence of a hydrophilic hydroxyl group on each carbon atom effectively neutralizes the hydrophobic character of the aliphatic chain, thereby rendering these glycols equivalent in effectiveness to the lowest unsubstituted homologue, methanol. Furthermore, this behavior is contrary to what one would expect if these glycols were perturbing the structure of the deoxy-Hb S polymer by competitive hydrogen bonding. Hence, interference with hydrogen bonds is not a controlling factor in destabilizing the polymer. (Because of their behavior, no assignment of n was made for either glycol in Table I.)

The effectiveness of straight-chain unsubstituted amides in enhancing solubility increased progressively according to the hydrocarbon content of the aliphatic moiety. The two isomers in the C_2 series, acetamide and N-methylformamide, both of which have n values of 1, were about equivalent in effectiveness. Similarly, for the C_3 series, propionamide was nearly as effective as N-ethylformamide, while N-methylacetamide was only slightly less so. The equipotency of propionamide and N-ethylformamide, while predicted by the concept of effective methylene units, is nevertheless remarkable inasmuch as the chemical nature of these two isomers is very different. It would appear from this behavior that the hydrophobic character of the ethyl group is equally well expressed whether it is attached to the carbonyl or the amino moiety of the amide. However, while N-methylacetamide was only slightly less effective than either of the preceding two C_3 isomers, N,N-dimethylformamide was, by contrast, considerably less effective than any of the other three C_3 isomers and was, in fact, only as effective as formamide itself in solubilizing deoxy-Hb S. It seems, therefore, that the substitution of a second methyl group on the amide nitrogen somehow impedes the expression of the first. (Because of this, no assignment of n has been made for this species in Table I.)

As was the case for both aliphatic alcohols and amides, the effectiveness of monalkyl-substituted straight-chain ureas was proportional to the length of the alkyl group. Similarly, the effect of branching of the alkyl side chain was analogous to that observed for alcohols; i.e., isobutylurea was essentially equipotent with propylurea (n = 3 in both cases). The results for the two dimethyl-substituted ureas are less clear-cut. While 1,3-dimethylurea was nearly as effective as ethylurea, 1,1-dimethylurea was only as effective as urea itself. By analogy to the amides, it would appear that the presence of a methyl group on both nitrogens is nearly equivalent to having a single ethyl group on either one, while the substitution of a second methyl group on any one nitrogen abolishes the effectiveness of the first. This being the case, it is not surprising then that tetramethylurea is neither more nor less effective than 1,1-dimethylurea. (Again, it was not possible to assign a meaningful value of n for either species in Table I.) It is also noteworthy that the effectiveness of hydroxyurea is essentially identical with that of urea. Thus, the presence of a hydroxyl group on the amide nitrogen has no effect on the intrinsic hydrophobic character of the urea.

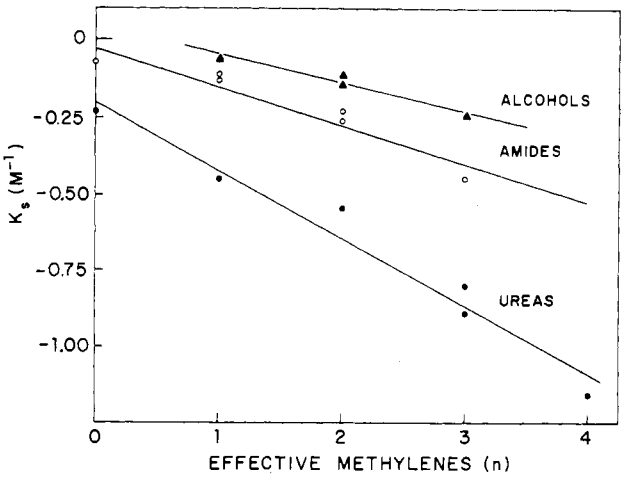

FIGURE 4: Plot showing the linear correlation between the Setschenow constant, K_s, and the number of effective methylene units, n, for each of the three classes of noncovalent inhibitors examined. Values of K_s were derived from the solubility data of Figures 1–3 as explained in footnote b to Table I.

Table II: Summary of Intercepts (K_{fn}) and Slopes ($K_{H\phi}$) Derived from the Linear Plots of K_s vs. n for Each Class of Perturbant[a]

perturbant class	K_{fn} (M^{-1})	$K_{H\phi}$ (M^{-1})	r[c]
alcohols	[b]	−0.095	0.978
amides	−0.028	−0.124	0.959
ureas	−0.200	−0.222	0.979

[a] $K_s = K_{fn} + nK_{H\phi}$. [b] Not relevant (see text). [c] Correlation coefficients for the linear plots of K_s vs. n shown in Figure 4.

For the most part, within each class of organic cosolvent studied, the effectiveness in increasing solubility was proportional to the hydrocarbon content of the aliphatic side chain, expressed as effective methylene units, n. Thus, in terms of intraclass comparisons, it is the hydrophobic character of the pendant alkyl moiety which determines the efficacy in perturbing the supramolecular structure of the deoxy-Hb S polymer.

A correlation has been made between K_s, the Setschenow constant, and n for the members of each perturbant class in order to facilitate cross-comparisons among the three classes of hydrophobic cosolvents. The respective plots of K_s vs. n for alcohols, amides, and ureas are shown in Figure 4. These plots are approximately linear in each case, and the data have been evaluated by linear regression analyses according to the equation

$$K_s = K_{fn} + nK_{H\phi} \quad (3)$$

In this treatment, K_s has been separated into two components, K_{fn}, the contribution due solely to the functional group (OH, $CONH_2$, or $NHCONH_2$), and $K_{H\phi}$, the contribution of the alkyl side chain per effective methylene group. Thus, for the three plots given in Figure 4, the intercept corresponds to K_{fn} and the slope to $K_{H\phi}$. The functionality parameter, K_{fn}, is meaningful only for amides and ureas and effectively corresponds to the value of K_s at n = 0. For alcohols, K_{fn} should be zero; i.e., the contribution of the OH group to K_s is nil. The parameter $K_{H\phi}$ is a measure of the intrinsic hydrophobicity per methylene group for a particular perturbant class. The appropriate values of K_{fn} and $K_{H\phi}$ derived from the data of Figure 4 are summarized in Table II.

The validity of dissecting the Setschenow constant into two separate, but additive, components, both of which reflect hydrophobic interactions with the solvent, is provided by the following considerations. Firstly, the values of K_{fn} for amides and ureas obtained from the data of Figure 4 (−0.03 and −0.20 M^{-1}, respectively) are in reasonable agreement with the experimentally determined values of K_s for formamide and urea (−0.07 and −0.25 M^{-1}, respectively). Secondly, the values of $K_{H\phi}$ are not constant, as might be expected if each methylene exerted a uniform effect regardless of the functionality to which it was attached. Instead, it appears that the functional group modulates the expression of the hydrophobic character of the pendant alkyl chain, the order of effectiveness being $NHCONH_2 > CONH_2 > OH$. Thirdly, the values of K_s calculated for the various hydrophobic perturbants (data not shown) by use of eq 3 and Table II are in reasonable agreement with the experimentally determined values listed in Table I. Hence, so long as an unambiguous value of n can be assigned, one may use eq 3, with the appropriate values of K_{fn} and $K_{H\phi}$, to obtain a reliable estimate of K_s for any aliphatic alcohol, amide, or urea not examined in this study.

It should be emphasized that the two components which comprise the Setschenow constant (K_{fn} and $K_{H\phi}$) in this treatment *both* reflect solvent-mediated hydrophobic effects. Polar contributions of the amide and urea functional moieties are negligible due to the low concentration of each species used in these studies (0.1–1.0 M). Since the individual contributions (K_{fn} and $K_{H\phi}$) to the Setschenow constant are solubilizing in either case, the net destabilization of the deoxy-Hb S polymer observed can be attributed to a composite solvent–protein interaction which is solely hydrophobic in origin. That is, these effects may be interpreted in terms of an alteration in the structure of water in the bulk solvent by the concerted action of both the nonpolar portion and the functional group of the cosolvent. Moreover, because the solubilizing effects of such hydrophobic perturbants are strictly solvent mediated, this treatment does not require the actual binding of these agents to specific sites on the macromolecule. This obviates any consideration of binding constants of the sort invoked by Herskovits et al. (1970a–c) for the isothermal denaturation of single-chain proteins, as well as tetrameric Hb A (Elbaum et al., 1974b), by the same chaotropic agents, but at considerably higher concentrations than those used in this study. Furthermore, the high concentrations of denaturants (2–16 M) used by Herskovits et al. (1970a–c) to effect complete unfolding required that both polar and nonpolar contributions of the organic additives to the supporting solvent be considered to explain the effects observed.

Studies which impinge more directly on this work were those in which the capacity of moderate concentrations (1–2 M) of alkylureas and aliphatic amides to promote the dissociation of Hb A from tetramer to dimers without denaturation was assessed (Elbaum & Herskovits, 1974; Bhat & Herskovits, 1975). In these studies, a correlation was observed between the degree of dissociation and the increasing chain length of the substituent alkyl group for either class of additive. In separate studies, Elbaum et al. (1974a, 1976) investigated the effects of straight-chain alkylureas on the solubility of deoxy-Hb S, as measured by MGC, the minimum gelling concentration (Bookchin & Nagel, 1971). Their results showed that gelation was inhibited by alkylureas at two different concentrations (0.1 and 0.2 M) to an extent that was roughly proportional to the length of the alkyl group. While this is in qualitative accord with our results, their findings were discrepant with ours for the two cases in which the equivalent of solubility profiles (MGC vs. ethyl- or propylurea concentration) were given. That is, their data were linear only at concentrations of >0.1 M; below 0.1 M a precipitous decline in MGC was observed. Furthermore, the slopes of the linear regions of their curvilinear plots were 17.2 and 26.0 g (dL $M)^{-1}$, values about half those of 29.3 and 45.9 g (dL $M)^{-1}$ found for ethyl- and propylurea, respectively, in our case (see Table I). The lack of correspondence between their data and ours for these additives can most likely be ascribed to the nonequilibrium conditions which prevail in the MGC assay.

The systematic evaluation of aliphatic alcohols, amides, and ureas reported here, as well as that of neutral salts reported earlier (Poillon & Bertles, 1979), clearly demonstrates the utility of such perturbants of hydrophobic interactions to destabilize the supramolecular structure of the deoxy-Hb S polymer. Furthermore, the individual Setschenow constants derived from such solubility data put the solvent-mediated approach to inhibiting gelation on a sound quantitative footing. This represents a significant advance over the more descriptive, less rigorous analyses hitherto published on the effects of various antisickling agents on the equilibrium solubility of deoxy-Hb S (Poillon & Bertles, 1977; Ross & Subramanian, 1977; Noguchi & Schecter, 1978; Behe & Englander, 1979). Moreover, the results of our studies considered in the aggregate compel one to conclude that ionic forces and hydrogen bonds play only a subsidiary role in stabilizing the deoxy-Hb S polymer. That is, the individual ion rankings demonstrated in our earlier study preclude nonspecific electrostatic shielding as the underlying basis for the effects of neutral salts on solubility, while the relative inertness of ethylene glycol and glycerol found in this study indicates the marginal contribution of hydrogen bonds to the overall stabilization energy. Further support for this conclusion is given by the equipotency of hydroxyurea and urea, as well as that of formamide and *N*,*N*-dimethylformamide, the latter being a species in which the dimethyl-substituted amide group cannot participate in hydrogen bonding. These data then tend to rule out competitive hydrogen bonding as a controlling factor in the solubilizing effects observed for amides and ureas. Instead, it would appear that the effectiveness of both lyotropic salts and aliphatic cosolvents results exclusively from the perturbation of intermolecular hydrophobic forces in the polymer.[5] Moreover, the fact that neither Hb A, Hb C (β6Glu → Lys; Bookchin & Nagel, 1974), Hb G Makassar (β6Glu → Ala; Blackwell et al., 1970), nor Hb Leiden (β6 or β7Glu → 0; Nagel et al., 1973) gels under conditions in which Hb S does (Bookchin & Nagel, 1974) clearly implicates the presence of valine at position β6 as the underlying cause of sickling in SS erythrocytes. This contention is buttressed by the recent demonstration (Magdoff-Fairchild & Chiu, 1979) that the X-ray diffraction pattern of deoxy-Hb S fibers bears sufficient resemblance to that of deoxy-Hb S crystals for the crystal structure, which is known to near atomic resolution (Wishner et al., 1975), to be validly used as a model for the polymer. In this case then, the principal noncovalent interaction stabilizing the double filament, which comprises the asymmetric unit of the crystal, is that between β6Val on one strand and the two nonpolar side chains of residues β85Phe and β88Leu on the other strand. This tripartite contact region is strongly

[5] This contention is further supported by the findings that urea, whose effectiveness as a protein denaturant arises largely from the destabilization of hydrophobic forces (Gordon & Jencks, 1963; Nozaki & Tanford, 1963) rather than from the breaking of hydrogen bonds (Levy & Magoulas, 1962), was nearly 4 times as effective as formamide (see K_s values in Table I) in solubilizing deoxy-Hb S.

hydrophobic and is, undoubtedly, the primary determinant of the intracellular polymerization which underlies the sickling phenomenon. Since this interaction contributes the bulk of the stabilization energy of the polymer, the deoxy-Hb S system effectively behaves as if only this key intermolecular contact were being perturbed by the addition of either lyotropic salts or organic additives. Such being the case, one approach to devising a suitable antisickling measure would be manipulation of the supporting solvent so as to destabilize this crucial contact. We have demonstrated such a solvent-mediated process for lyotropic salts (Poillon & Bertles, 1979) and now for organic nonelectrolytes. Moreover, having established the principle of additivity for the component parts of both lyotropic salts and aliphatic cosolvents, one would expect that the effectiveness of other types of noncovalent perturbants in increasing the solubility of deoxy-Hb S might also be expressed as an approximately additive function of their constituent groups. Thus, it should be possible to establish an index of solubility parameters which would permit the overall K_s value for any compound being considered as a potential antisickling agent to be calculated simply as the sum of the K values for its component parts. This approach would provide a rational basis for the design of antisickling agents in which the optimum potential for inhibition of gelation could be achieved by maximizing the overall K_s values. The feasibility of using lyotropic salts and organic nonelectrolytes, either separately or in combination, in a therapeutically useful regimen for the treatment of sickle cell anemia would thereby be considerably enhanced.

Added in Proof

It has come to our attention that a study of the effects of a variety of inorganic and organic additives on the solubility of deoxy-Hb S has already appeared (Ross & Subramanian, 1978). For the most part, the results obtained in that study were in reasonable agreement with ours for those compounds evaluated in common. No attempt was made, however, in this earlier report to obtain a quantitative measure of molar effectiveness for the additives examined.

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