Noncovalent Inhibitors of Sickle Hemoglobin Gelation: Effects of Aryl-Substituted Alanines†

William N. Poillon

With the Technical Assistance of Bak C. Kim

ABSTRACT: The effects of 42 β-aryl-substituted alanines on the inhibition of gelation of sickle hemoglobin (Hb S) were evaluated. These included 18 derivatives of phenylalanine, 16 derivatives of tryptophan, and 4 analogues of each of these aromatic amino acids. Among the para-halogen-substituted phenylalanine derivatives, the order of molar effectiveness in increasing the solubility of deoxy-Hb S was I ~ Br > Cl > F; substituents at the equivalent 5 position of the indole ring of tryptophan gave the order Br > OCH_3 ~ OH > CH_3 > F. For three phenylalanine derivatives substituted on the alanyl side chain by a methyl group, the order was N-CH_3 > α-CH_3 ≫ β-CH_3. A similar situation prevailed for the N-CH_3 and α-CH_3 derivatives of tryptophan. For two other para-substituted phenylalanines the nitro derivative was more effective than the amino derivative by nearly 3-fold. For the analogues of phenylalanine, β-(1-naphthyl)alanine was 2.5 times more effective than β-(2-naphthyl)alanine, while β-(2-thienyl)alanine and β-(3-thienyl)alanine were equipotent and somewhat less effective than phenylalanine. All three analogues of tryptophan were more potent than the parent compound and showed the following order of effectiveness: 7-azaindol-3-yl > 3-benzothienyl > 3-benzofuranyl. From the overall results obtained, the following generalizations can be made regarding the efficacy of β-aryl-substituted alanines as noncovalent inhibitors of gelation: (1) bicyclic aromatic nuclei are considerably more potent than monocyclic ones and (2) the nature of the substituent at a fixed position on a particular aromatic ring exerts a profound effect on the expression of antigelling activity. The best gelation inhibitor to emerge from this comprehensive survey was 5-bromotryptophan, which was 5 times more effective than phenylalanine. By suitable manipulation of either the aromatic nucleus or the alanyl side chain, it should be possible to increase antigelling activity to a level potent enough to be useful in a therapy for sickle cell anemia.

The molecular events which underly the pathophysiology of sickle cell anemia result from the substitution of valine for glutamic acid at position 6 in the β chain of sickle hemoglobin (Hb S).[1] Upon deoxygenation of erythrocytes from patients homozygous for Hb S (SS erythrocytes), polymers of deoxy-Hb S are formed which spontaneously align into paracrystalline arrays that distort the cell into the variety of bizarre shapes which are ultimately responsible for occlusion of the microcapillaries.

The in vitro correlate of such intracellular polymerization is the gelation of hemolysates of SS erythrocytes (or purified Hb S) observed when the solubility of deoxy-Hb S is exceeded, i.e., the concentration above which monomers are in equilibrium with polymers. The separation of this semisolid gel into its two component phases by ultracentrifugation provides a means by which the equilibrium solubility or saturation concentration, c_{sat}, can be measured (Hofrichter et al., 1976; Magdoff-Fairchild et al., 1976; Poillon & Bertles, 1977; Dean & Schechter, 1978; Poillon & Bertles, 1979; Poillon, 1980). This technique has been used widely to assess the effects of variables such as pH, temperature, and initial concentration, as well as potential antigelling agents, on this two-phase equilibrium.

Because the predominant noncovalent forces which stabilize the supramolecular structure of the deoxy-Hb S polymer are strongly hydrophobic, our approach to the design of an effective antisickling agent has been to manipulate the supporting solvent in such a manner as to inhibit gelation. We have already established the validity of this approach for both lyotropic salts (Poillon & Bertles, 1979) and three classes of organic additives [aliphatic alcohols, amides, and ureas (Poillon, 1980)]. In the latter case it was further shown that molar effectiveness depended on both the length of the aliphatic side chain and the nature of the functional group to which it was attached, according to the order $NHCONH_2$ > $CONH_2$ > OH. This being the case, our original intention was to extend this comprehensive survey to include arylureas. However, due to the limited water solubility of such compounds, this was not feasible. Instead, we turned to aryl-substituted alanines. It has been shown by others (Ross & Subramanian, 1977, 1978; Noguchi & Schechter, 1978; Behe & Englander, 1979) that among the amino acids, those with aromatic side chains (tryptophan and phenylalanine) were considerably more effective than any of the aliphatic ones. Accordingly, the present study was undertaken in order to determine whether the antigelling capacity of these two amino acids could be potentiated still further by suitable substitution on the respective aromatic ring (indole or benzene); certain analogues of these two aryl moieties were evaluated as well.

Experimental Procedures

Sickle Hemoglobin. Two different types of patients homozygous for sickle cell anemia were used as the source for Hb S: (1) those who were asymptomatic and clinically stable in a steady state and (2) those who were hospitalized and had been partially exchange transfused at some point in the recent past. In either case, hemolysates were obtained from 3 times washed erythrocytes as previously described (Magdoff-Fairchild et al., 1976). Because the erythrocytes of patients from category 1 contained <5% fetal hemoglobin, hemolysates obtained therefrom could be used directly without further

† From the Center for Sickle Cell Disease and Department of Pediatrics and Child Health, Howard University, Washington, D.C. 20059. *Received August 20, 1981.* Supported by Center Grant HL 15160 from the National Institutes of Health, U.S. Public Health Service.

[1] Abbreviations: Hb S, sickle hemoglobin; c_{sat}, saturation concentration; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; SS erythrocytes, erythrocytes from patients homozygous for Hb S; Hb F, fetal hemoglobin; Hb A, adult hemoglobin; Hb A_2, minor hemoglobin component of normal adult blood.

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purification (Poillon & Bertles, 1979; Poillon, 1980). However, because the erythrocytes from patients in category 2 usually contained from 20 to 40% adult hemoglobin, Hb S was purified by ion-exchange chromatography, using an adaptation of the method of Huisman & Dozy (1965) in which DEAE-Sephacel was substituted for DEAE-Sephadex A-50. The lysate, which had been dialyzed against 0.01 M Tris buffer (pH 8.30 at 25 °C), was applied to a column (5 × 42 cm) preequilibrated in the cold with 0.05 M Tris buffer (pH 8.20 at 25 °C). Each of the four component hemoglobins (A_2, S, F, and A) was sequentially eluted with a pH gradient of 0.05 M Tris buffer covering the range 8.2–7.3. The Hb S fraction was concentrated to 32–36 g/dL by vacuum ultrafiltration, as was the lysate from category 1. In either case, samples were extensively dialyzed against 0.1 M Bis-Tris buffer (pH 6.8 at 30 °C) in the cold, prior to use.

Derivatives and Analogues of Phenylalanine and Tryptophan. For the most part, the various aryl-substituted alanine derivatives used in this study were obtained from various commercial sources and used without further purification. β-(3-Benzofuranyl)alanine was provided by Dr. Albert A. Manian, Neurosciences Research Branch, NIMH; β-(3-benzothienyl)alanine and its 5-hydroxy derivative were provided by Dr. Talmadge R. Bosin, University of Indiana. Because many of the compounds evaluated were only sparingly water soluble at neutral pH, it was necessary to convert them to their sodium salts. This was achieved by titration of a slurry of the compound in distilled water to pH 10–11 with 1 N NaOH. For stock solution concentrations in the range 0.06–0.24 M, this treatment resulted in complete dissolution of the material; the maximum amount of alkali used to achieve solution in each case was sufficiently small so that the pH of the resultant solution of Hb S plus additive never exceeded the upper limit of the pH range in which we have found c_{sat} to be essentially invariant (pH 6.20–6.85).

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 240-μL total volume were centrifuged for 1 h at 242000*g* in an SW 50.1 rotor at 30 °C. After centrifugation to separate the two component phases of the gel, the pH and concentration of the supernatant phase (monomeric Hb S) were measured in the usual manner (Magdoff-Fairchild et al., 1976). Conversion of oxy- to deoxy-Hb S was achieved with a 3-fold molar excess of sodium dithionite; the initial concentration of Hb S was in the range of 25–29 g/dL. Because of the variable dilution incurred by the addition of the various compounds evaluated, the final concentration of Bis-Tris buffer ranged from 63 to 82 mM.

Results

Base-Line Solubility. Overall, the effects of 42 β-aryl-substituted alanines on the equilibrium solubility, c_{sat}, of deoxy-Hb S were evaluated. All species examined were derivatives or analogues of either phenylalanine or tryptophan. In each case, a control sample (in the absence of additive) was included. The Hb S was obtained from hemolysates of either SS erythrocytes, in which case it was used directly, or SA erythrocytes, in which case purification by ion-exchange chromatography (as previously described) was required. For each source, the solubility data accumulated for such controls were separately subjected to statistical analysis. A value of c^0_{sat} = 19.8 ± 0.9 g/dL (n = 18) was obtained for hemolysates of SS erythrocytes, while purified Hb S gave a value of 19.1 ± 0.4 g/dL (n = 23). While the standard deviation in the former case was roughly twice as large as in the latter, the two mean values of c^0_{sat} are nearly identical. Hence, one may use either source of Hb S interchangeably to obtain comparable solubility data for any particular additive.

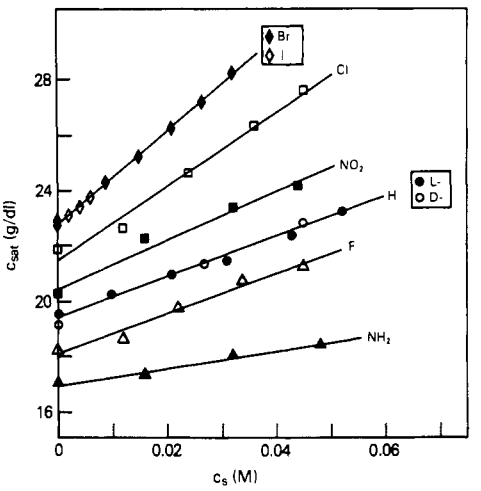


FIGURE 1: Solubility profiles for para-substituted phenylalanine derivatives in which the equilibrium solubility of deoxy-Hb S, c_{sat}, has been plotted against the concentration of additive, c_s. In all cases, Hb S samples were equilibrated for 1 h, and phase separation was achieved by centrifugation at 242000*g* for 1 h at 30 °C. The concentration of Hb S in the supernatant phase corresponds to c_{sat}. (It should be noted that the solubility profiles shown in Figures 1–4 have been, in some cases, arbitrarily displaced on the *y* axis for the purpose of clarity; this maneuver in no way affects the value of the slope of such plots. The mean value of 19.5 g/dL, obtained as described in the text, may be considered the reference value of c^0_{sat} for all species examined.)

Table I: Solubility Parameters for Derivatives of Phenylalanine

species	slope [g/(dL·M)]	R^a
L-phenylalanine	73	1.00
D-phenylalanine	73	1.00
o-fluorophenylalanine	55	0.75
m-fluorophenylalanine	68	0.93
p-fluorophenylalanine	72	0.99
o-chlorophenylalanine	110	1.51
p-chlorophenylalanine	134	1.84
p-bromophenylalanine	166	2.27
p-iodophenylalanine	165	2.26
p-aminophenylalanine	31	0.42
p-nitrophenylalanine	87	1.19
α-methylphenylalanine	78	1.07
β-methylphenylalanine	46	0.63
N-methylphenylalanine	89	1.22
N-carbamoylphenylalanine[b]	69	0.95
3-nitrotyrosine	160	2.19
3-iodotyrosine	99	1.36
DBA[c]	78	1.07

[a] This parameter corresponds to the ratio of the slopes of the solubility profile for each derivative to that for the unmodified species, phenylalanine or tryptophan. [b] Synthesized from phenylalanine by the procedure of Behe & Englander (1979). [c] 3,4-Dihydro-2,2-dimethyl-2*H*-1-benzopyran-6-butyric acid.

Phenylalanine Derivatives. Solubility profiles (c_{sat} vs. concentration, c_s) for various para-substituted derivatives of phenylalanine are shown in Figure 1. The corresponding values of the slopes for these plots, as well as the ratios (R) of each slope to that for unsubstituted phenylalanine, are compiled in Table I. Inasmuch as the magnitude of the slope is directly proportional to the molar effectiveness as an inhibitor of gelation, these ratios represent the increment (R > 1) or decrement (R < 1) in solubility evoked by each species, relative to that of the parent compound, phenylalanine. It is

noteworthy that there was no significant difference in capacity to inhibit gelation for the two stereoisomers of phenylalanine. Accordingly, the solubility data for the L and D isomers were plotted on a single line (see Figure 1).

Among the para-halogen-substituted derivatives of phenylalanine, the order of molar effectiveness was

$$I \sim Br > Cl \gg F$$

From the values of R given in Table I, it is evident that the p-iodo and p-bromo derivatives were essentially equipotent (R = 2.3) while the p-chloro derivative was somewhat less potent (R = 1.8). By contrast, the p-fluoro derivative was considerably less effective, being essentially equipotent with phenylalanine itself (R = 1.0). Although their solubility profiles are not shown, the effects of halogen substitution at the ortho and meta positions of the phenyl group are also provided in Table I. It appears that meta substitution is roughly equivalent to para substitution, at least for the fluorine atom. For both fluorine and chlorine, however, the ortho-substituted derivative is significantly less effective than the para-substituted one, eliciting a diminution in molar effectiveness of about 25% in either case (cf. values of slope in Table I). For the two other para-substituted derivatives depicted in Figure 1, the order of molar effectiveness was $NO_2 \gg NH_2$. While the p-nitro derivative is marginally more effective than phenylalanine itself (R = 1.2), the p-amino derivative is less than half as effective (R = 0.4). Furthermore, relative to each other, the p-nitro derivative is more potent than the p-amino derivative by nearly 3-fold.

Table I also includes solubility data for three methyl-substituted derivatives (whose solubility profiles are not shown) in which substitution occurs at various positions on the alanyl side chain rather than on the benzene ring. The order of molar effectiveness was

$$N\text{-}CH_3 > \alpha\text{-}CH_3 \gg \beta\text{-}CH_3$$

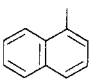
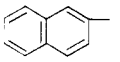
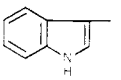
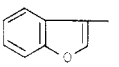
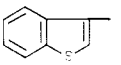
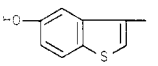
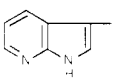
The N-methyl derivative is only slightly more effective than phenylalanine (R = 1.2), while the α-methyl derivative is essentially equipotent (R = 1.1) with it. By contrast, the β-methyl derivative is considerably less effective than phenylalanine (R = 0.6). Relative to each other, the α-methyl derivative is about 1.7 times more effective than the β-methyl one, suggesting steric hindrance when methyl substitution occurs at the carbon atom adjacent to the benzene ring.

In the case of N-carbamoylphenylalanine, the R value of 0.95 indicates that it is only marginally less effective than phenylalanine. It is also noteworthy that this species is nearly 25% less effective than the N-methyl derivative.

Another species related to phenylalanine which has been included in Table I is DBA [3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-6-butyric acid (Poillon & Bertles, 1977)]. It may be seen from the magnitude of its slope that it is essentially equipotent with phenylalanine and, therefore, of little value as a potential antisickling agent.

Although it was not possible to evaluate tyrosine directly because of its limited water solubility, we were able to examine two of its derivatives (see Table I). From their respective R values, it may be seen that both derivatives were more potent than phenylalanine. Furthermore, relative to each other, 3-nitrotyrosine was about 1.6 times more effective than 3-iodotyrosine. This behavior contrasts sharply with that shown by the corresponding para-substituted phenylalanines where the p-nitro derivative was only half as effective as the p-iodo derivative. These findings serve to illustrate another important feature of the structure–antigelling activity correlations which we have discerned from this comprehensive survey, namely, the importance of the position of the substituent on the benzene ring. When substitution occurs at the para position, the iodo derivative is considerably more effective than the nitro (by nearly 2-fold). However, the reverse situation occurs with substitution at the meta position; i.e., the nitro derivative is more potent than the iodo derivative by a considerable margin (more than 1.6-fold).[2]

Table II: Solubility Parameters for Analogues of Phenylalanine and Tryptophan

β-aryl substituent	X[a]	slope [g/(dL·M)]	R[b]
(A) Phenylalanine Analogues			
phenyl		73	1.00
1-naphthyl		176	2.41
2-naphthyl		67	0.92
2-thienyl		55	0.75
3-thienyl		55	0.75
(B) Tryptophan Analogues			
3-indolyl		153	1.00
3-benzofuranyl		171	1.12
3-benzothienyl		200	1.31
5-hydroxy-benzothien-3-yl		186	1.22
7-azaindol-3-yl		213	1.39

[a] These compounds are considered on the basis of the general structural formula for a β-aryl-substituted alanine: $X\text{-}CH_2CH(NH_2)COOH$. [b] See footnote *a* of Table I.

Phenylalanine Analogues. The effects of replacement of the benzene ring of phenylalanine by two other aromatic ring systems (thienyl and naphthyl) on the solubility of deoxy-Hb S are shown in Figure 2. The corresponding solubility data, as well as the aromatic ring structures for these analogues of the benzene ring of phenylalanine, are shown in part A of Table II. For the five-membered heterocyclic thienyl ring

[2] It is recognized that these solubility data are not strictly comparable because we have ignored the contribution of the hydroxyl group at the para position to this phenomenon. However, since the potency of tyrosine relative to that of phenylalanine cannot be evaluated, we feel justified in doing this on the basis of the two cases in our study (Tables II and III) in which the hydroxyl group was substituted at the 5 position of a heterocyclic aromatic nucleus. For 5-hydroxytryptophan, the activity was increased about 1.4-fold relative to that of tryptophan, while for 5-hydroxy(3-benzothienyl)alanine, it was suppressed slightly relative to that of the sulfur analogue of tryptophan. Inasmuch as we have shown that the 5 position of the indole ring is equivalent in reactivity to the para position of benzene, one would predict that tyrosine would at best be only slightly more effective than phenylalanine and that the hydroxyl group contributes marginally to the overall antigelling activity.

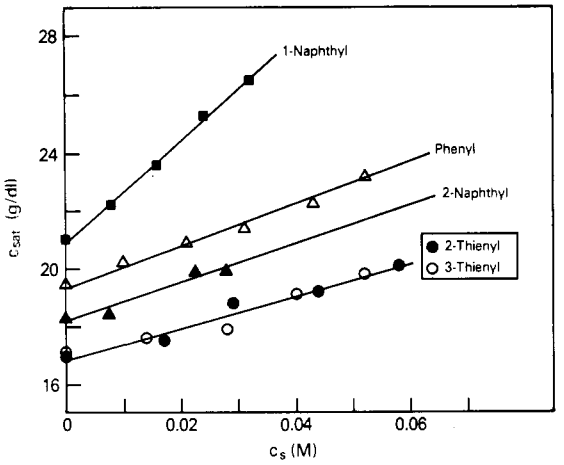


FIGURE 2: Solubility profiles for phenylalanine analogues (denoted in terms of the aryl substituent on the β carbon of alanine) in which the equilibrium solubility of deoxy-Hb S, c_{sat}, has been plotted against the concentration of additive, c_s. Other experimental conditions are given in the legend to Figure 1.

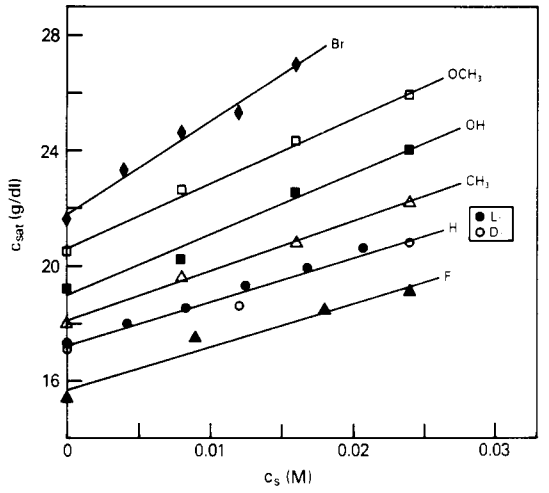


FIGURE 3: Solubility profiles for tryptophan derivatives, substituted at the 5 position of the indole ring, in which the equilibrium solubility, c_{sat}, has been plotted against the concentration of additive, c_s. Other experimental conditions are given in the legend to Figure 1.

system, attachment to the β carbon of alanine at either position 2 or 3 made no difference—both were equipotent and about 25% less effective than phenylalanine. By contrast, a striking difference was observed for the two 10-membered bicyclic naphthyl ring systems. In this case, attachment to the β carbon of alanine at position 1 of the naphthalene ring resulted in a species which was nearly 3 times more effective than attachment of the naphthalene ring at position 2. The latter derivative was essentially equipotent with phenylalanine (R = 0.9), while the former was nearly 2.5 times more effective (R = 2.4).

Tryptophan Derivatives. The effects of various 5-substituted tryptophan derivatives on the solubility of deoxy-Hb S are shown in Figure 3. As was the case for phenylalanine, the two stereoisomers of tryptophan were equipotent, and the combined solubility data for the L and D isomers were plotted on a single line. The overall order of molar effectiveness was

$$Br > OCH_3 \sim OH > CH_3 > F$$

In order to facilitate more informative comparisons, this ranking will be further broken down into subsets, the solubility data for which are given in Table III. The most salient feature of these data is that the 5-bromo derivative was twice as effective as the 5-fluoro derivative, which in turn was equipotent with tryptophan (cf. respective R values in Table III). This same relative rank order was also observed for the *p*-bromo- and *p*-fluoro-substituted phenylalanine derivatives (cf. corresponding values of R in Table I), indicating that the para position of the benzene ring of phenylalanine is equivalent in reactivity to the 5 position of the indole ring in tryptophan. Of the remaining three derivatives depicted in Figure 3, 5-methoxytryptophan and 5-hydroxytryptophan were nearly equipotent, and both were slightly more effective than 5-methyltryptophan; each was slightly to moderately more effective than tryptophan itself ($R \leq 1.5$ in all three cases).

Table III: Solubility Parameters for Derivatives of Tryptophan

species	slope [g/(dL·M)]	R^a
L-tryptophan	153	1.00
D-tryptophan	153	1.00
4-fluorotryptophan	141	0.92
5-fluorotryptophan	159	1.04
6-fluorotryptophan	178	1.16
5-bromotryptophan	320	2.09
5-hydroxytryptophan	213	1.39
5-methoxytryptophan	224	1.46
1-methyltryptophan	123	0.80
5-methyltryptophan	174	1.14
6-methyltryptophan	176	1.15
7-methyltryptophan	190	1.24
α-methyltryptophan	130	0.85
N-methyltryptophan	180	1.18
tryptamine	108	0.71
3-indolepropionic acid	80	0.52

[a] See footnote *a* of Table I.

The effects of substitution with fluorine at the 4, 5, and 6 positions of the indole ring of tryptophan (solubility profiles not shown) are indicated in Table III. Small, but significant, differences were found for these three derivatives, with the following order of molar effectiveness:

$$6\text{-F} > 5\text{-F} > 4\text{-F}$$

Table III also includes solubility data for six methyl-substituted derivatives of tryptophan (solubility profiles not shown). For those in which substitution occurs at different positions on the indole ring, the order of molar effectiveness was

$$7\text{-}CH_3 \sim 6\text{-}CH_3 \sim 5\text{-}CH_3 > 1\text{-}CH_3$$

Placement of the methyl group at position 5, 6, or 7 of the indole ring results in species which are essentially equipotent and only slightly more effective than tryptophan ($R \leq 1.2$). Its placement at position 1, the nitrogen atom of the pyrrole moiety of the indole ring, results in a species which is significantly less effective than tryptophan (R = 0.8), indicating the importance of a hydrogen atom at this position to the maximal expression of antigelling activity. For the two methyl-substituted derivatives in which substitution occurs at either the α carbon or the amino nitrogen of the alanyl side chain, the order of molar effectiveness was

$$N\text{-}CH_3 > \alpha\text{-}CH_3$$

Substitution at the nitrogen atom enhances antigelling activity slightly (R = 1.2), while substitution at the α carbon reduces it slightly (R = 0.85), relative to that of tryptophan. It is also noteworthy that the analogous substitution at the nitrogen atom for phenylalanine potentiates the antigelling activity to the same extent (~20%) as that for tryptophan (cf. appropriate values of slopes in Tables I and III).

Tryptophan Analogues. The effects of three analogues of tryptophan, as well as a derivative of one of them, on the solubility of deoxy-Hb S are shown in Figure 4. All three

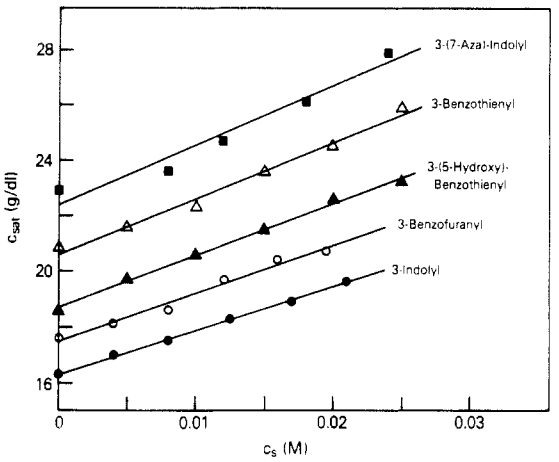


FIGURE 4: Solubility profiles for tryptophan analogues (denoted in terms of the aryl substituent on the β carbon of alanine) in which the equilibrium solubility of deoxy-Hb S, c_{sat}, has been plotted against the concentration of additive, c_s. Other experimental conditions are given in the legend to Figure 1.

analogues were more effective than tryptophan, according to the order 7-azaindol-3-yl > 3-benzothienyl > 3-benzofuranyl > 3-indolyl. The derivative (5-hydroxybenzothien-3-yl)alanine was essentially equipotent with its parent compound, (3-benzothienyl)alanine. (The aromatic ring structures corresponding to these analogues of the indole ring of tryptophan are given in part B of Table II). The most effective analogue, (7-azaindol-3-yl)alanine, in which a nitrogen atom is inserted at position 7 of the indole ring, enhanced the antigelling activity by a factor of 1.4-fold relative to that of tryptophan. The order of molar effectiveness with respect to the nature of the atom at position 1 of the pyrrole moiety of the indole ring was S > O > N. This is the order one would predict on the basis of the polarizability of these atoms and their position in the periodic table.

Because amino acids exist as zwitterions at neutral pH, we assessed the effect of removal of either the carboxyl or the amino group from the α carbon of the two parent amino acids. While the results were qualitatively in accord for both amino acids, those for tryptophan were more definitive and are summarized in Table III. It is evident that removal of either the carboxyl group (tryptamine) or the amino group (3-indolepropionic acid) reduces the antigelling activity to as much as half of its original value. Thus, the presence of both charged groups on the α carbon appears to be essential to the expression of maximal antigelling potency of the indolyl group appended to the β carbon of alanine.

Discussion

The comprehensive study of the effects of amino acids and oligopeptides on the gelation of Hb S reported by others (Noguchi & Schechter, 1978) showed that only tryptophan and phenylalanine were moderately effective inhibitors of gelation and that tryptophan was roughly twice as effective as phenylalanine. The solubility data presented in Tables I and III confirm, in more quantitative fashion, that the antigelling capacity of tryptophan is indeed greater than that of phenylalanine by a factor of 2.1-fold (cf. ratio of slopes for these two species). Furthermore, because alanine itself has no effect on gelation (solubility data not shown), the inhibition observed may be attributed solely to the aryl substituent on the β carbon of alanine (indole and benzene, respectively). The potency of the inhibition observed for the various derivatives and analogues of these two aromatic amino acids that are compiled in Tables I–III may be accounted for similarly. The solubility profiles (c_{sat} vs. c_s) presented in Figures 1 and 3 show also that for either phenylalanine or tryptophan the L and D stereoisomers are equipotent in inhibiting the polymerization of deoxy-Hb S. Hence, there appears to be no stereospecificity requirement associated with the perturbation of the gelation process, as has also been reported by others (Noguchi & Schechter, 1978; Gorecki et al., 1980).

In the present study, a total of 42 β-aryl-substituted alanines were evaluated for their ability to inhibit the gelation of deoxy-Hb S by noncovalent interactions. These compounds could be arranged into four categories: (1) para-substituted phenylalanine derivatives; (2) bicyclic and heterocyclic analogues of phenylalanine; (3) 5-substituted tryptophan derivatives; (4) heterocyclic analogues of tryptophan. Informative comparisons within each of these four categories were facilitated by calculation of the ratio of the slope of the solubility profile for any particular additive to that for the parent species (either phenylalanine or tryptophan, respectively). This parameter, R, is a convenient index of the extent to which the intrinsic antigelation activity is either augmented or diminished by any particular modification of the aromatic nucleus attached to the β carbon of alanine. The following general conclusions may be drawn from the results presented in Tables I–III.

(1) Overall, for the phenyl ring, the best derivative was p-bromophenylalanine, while the best analogue was (1-naphthyl)alanine, both species showing an enhancement in antigelling capacity of over 2-fold relative to that of phenylalanine. Likewise, for the indolyl ring, the best derivative was 5-bromotryptophan, while the best analogue was (7-azaindol-3-yl)alanine, each species potentiating the antigelling activity by factors of 2.1- and 1.4-fold, respectively, relative to that of tryptophan (see appropriate R values in Tables I–III).

(2) The nature of the aryl substituent on the β carbon of the alanyl residue affects the antigelling activity to varying degrees. For the most part, those composed of bicyclic aromatic ring systems are considerably more effective than those which are monocyclic. This is most vividly demonstrated by the nearly 2.5-fold potentiation of antigelling activity shown by (1-naphthyl)alanine relative to that of phenylalanine. By contrast, there is virtually no difference in antigelling activity between (2-naphthyl)alanine and phenylalanine. It is not clear why there is such a profound differential response to the ring position at which the naphthyl group is attached to the β carbon since both species are coplanar and have 10 π electrons in the aromatic nucleus. Among the four analogues of tryptophan examined, the 7-azaindole ring was most potent, being nearly 3 times as effective as phenylalanine. Thus, heterocyclic aromatic ring systems are, as a rule, considerably more potent inhibitors of gelation than are monocyclic ones (cf. magnitude of slopes for 3-benzothienyl vs. 3-thienyl in Table II). Furthermore, for the two analogues in which the nitrogen atom of the pyrrole ring of indole was replaced by either sulfur or oxygen, the order of molar effectiveness was S > O > N. This ranking corresponds to the order of decreasing polarizability for these three atoms. On this basis, one would predict, therefore, that the sulfur analogue of 7-azatryptophan should be roughly 1.3 times more effective than the parent compound [see the R value for (3-benzothienyl)alanine in Table II]. Unfortunately, this prediction cannot as yet be tested because this compound is not available commercially.

(3) The nature of the substituent at a fixed position on a particular aromatic ring also exerts profound effects on the expression of antigelling activity. The extent to which this is so is best illustrated by the para-substituted derivatives of phenylalanine, the solubility data for which are given in Table

I. Among the halogenated derivatives, the *p*-bromo one was over twice as effective as the unsubstituted amino acid,[3] while the *p*-fluoro one was equipotent with it. This differential behavior is what one would predict on the basis of both the polarizability and the steric bulk of the halogen atom. Another revealing comparison is that between the nitro and amino derivatives. While the nitro derivative enhanced the antigelling activity only marginally, it is nearly 3 times as effective as the amino derivative. In this case, the widely disparate influence of these two groups on the inhibition of gelation might be related to their opposing resonance effects in electrophilic substitution reactions (Pauling, 1960). It is not at all clear, however, why the ortho–para-directing, electron-donating amino group should evoke such a drastic diminution in antigelling capacity, relative to the meta-directing, electron-withdrawing nitro group. This phenomenon can only be documented as an empirical observation at this point.

(4) With respect to the therapeutically useful range for noncovalent inhibitors of gelation, it has been argued that the kinetically important delay time of gelation, t_d, relative to the capillary transit time, is the critical variable determining clinical severity in sickle cell anemia (Eaton et al., 1976; Sunshine et al., 1979a,b). This kinetic parameter is related to the equilibrium solubility, c_{sat}, by the empirical supersaturation equation in which the ratio $(c_i/c_{sat})^n$ occurs, where c_i is the initial concentration and n has a value of $\sim$35. This tremendous power dependence of the delay time on the supersaturation ratio makes it exquisitely sensitive to small changes in c_{sat}. We can, therefore, ask the question: "To what extent must the delay time be increased to result in a therapeutically beneficial effect"? It has been shown (Sunshine et al., 1978) that an increase in t_d of 10–100-fold would effectively ameliorate the clinical symptomatology to that of the less severe form of sickle cell disease, S/β^+-thalassemia. By use of the supersaturation equation (Hofrichter et al., 1976), we have calculated that a 10% increase in the equilibrium solubility would correspond to a 30-fold increase in t_d; i.e., it would be within the range predicted for minimal therapeutic benefit (Sunshine et al., 1979a,b). Accordingly, we have replotted the solubility data for the six most potent inhibitors, as well as for phenylalanine and butylurea, in terms of the solubility ratio (c_{sat}/c^0_{sat}) as a function of concentration, c_s. The results are shown in Figure 5. From these plots, one can interpolate the concentration of additive necessary to evoke an increase of 10% in c_{sat} (i.e., that corresponding to $c_{sat}/c^0_{sat} = 1.10$). Arranged in order of decreasing potency, the values are as follows: 5-bromotryptophan, 6 mM; 7-azatryptophan, 8 mM; (3-benzothienyl)alanine, 10 mM; *p*-bromophenylalanine, (1-naphthyl)alanine, and tryptophan, which are essentially equipotent and have been plotted on a single line, 12 mM; phenylalanine, 30 mM. Thus, 5-bromotryptophan is 5 times more potent than phenylalanine, which in turn is only marginally more effective than butylurea, the best aliphatic noncovalent inhibitor evaluated to date (Elbaum et al., 1974; Poillon, 1980). There are three aryl substituents which impart an antigelling capacity significantly greater than that of just the indolyl group of tryptophan. These are, in order of decreasing potency: 5-bromoindolyl, 7-azaindolyl, and benzothienyl. By analogy with the phenyl and indolyl groups, in which appropriate substitution with bromine on the aromatic nucleus results in a further enhancement of antigelling activity by approximately 2-fold, one would predict that the identical

[3] Although the *p*-iodo derivative was equally effective, its sparingly low water solubility renders it unsuitable for further consideration as a potential antisickling agent.

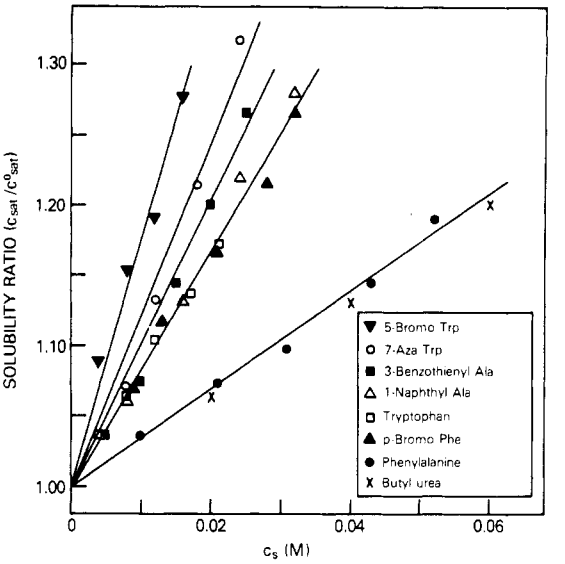


FIGURE 5: Relative solubility (c_{sat}/c^0_{sat}) of deoxy-Hb S as a function of additive concentration, c_s, for the most potent β-aryl-substituted alanines examined. The two terms in the ratio plotted on the ordinate represent the solubility in the absence (c^0_{sat}) and presence (c_{sat}) of additive, whose concentration is given on the abscissa. The lower the concentration of additive needed to evoke a fixed increment in solubility, the greater its capacity to inhibit gelation.

halogen substitution on the latter two aromatic nuclei should result in a similar increase in potency. Thus, e.g., in the case of the 7-azaindolyl moiety, bromination at the 5 position should result in a noncovalent inhibitor nearly 3 times as effective as tryptophan, i.e., one which would increase the solubility of deoxy-Hb S by 10% at a concentration of $\sim$4 mM. This concentration is near that for intracellular hemoglobin, suggesting a close to stoichiometric interaction, i.e., one in which the inhibitor would be tightly bound. Thus, the 5-bromo-7-azaindolyl derivative represents a species which should be at least 6 times more effective than phenylalanine. Depending on its water solubility and red cell permeability, one could conceivably achieve an intracellular concentration in the range of 4 mM by equilibration of SS erythrocytes to a steady state. This degree of antigelling potency should be sufficient to interfere with the intracellular polymerization of deoxy-Hb S, thereby ameliorating the painful symptoms of this debilitating disease to the extent outlined above (Sunshine et al., 1978).

A series of studies which impinge directly on the results presented here has been reported elsewhere (Ross & Subramanian, 1977, 1978; Noguchi & Schechter, 1978; Behe & Englander, 1979; Gorecki et al., 1980). These authors have conducted systematic surveys of the effects on solubility of a variety of aromatic compounds including amino acids, phenyl and other aryl derivatives, and oligopeptides containing various aromatic and polar side chain residues. Overall, these studies have revealed that the most efficient noncovalent inhibitors of gelation contain an aromatic nucleus with a pendant aliphatic side chain terminating in a functional group (amino, carboxyl, or hydroxyl) which possesses hydrogen-bonding capacity. In one case (Ross & Subramanian, 1977, 1978), the results have been used to formulate a mechanism for the inhibition of gelation by such compounds, based upon the reported intermolecular contacts in the deoxy-Hb S crystal (Wishner et al., 1975), which presumably are identical in the gel (Magdoff-Fairchild & Chiu, 1979). The principal noncovalent interactions that stabilize the gel are those side-to-side contacts within the double-stranded units of Hb S molecules which comprise the polymer. These consist of a strongly hydrophobic, tripartite contact between the mutation site

β6Val on one strand and the two nonpolar side chains of residues β85Phe and β88Leu on the other strand. Additionally, there is a hydrogen-bond contact between β4Thr on the strand containing the mutation site and β73Asp on the other strand. This stabilizing lateral contact region between two β chains in the double strand of the deoxy-Hb S polymer may be perturbed to varying extents by noncovalent inhibitors with the general structural formula ϕ–$(CH_2)_n$–Z, where ϕ represents the aromatic nucleus and Z the appropriate functional group ($-NH_3^+$, $-COO^-$, or –OH), separated by a number of intervening methylene groups, n. The destabilizing effect of the inhibitor would then result from its being competitively bound to the β6Val site by hydrophobic interaction with the nonpolar aromatic nucleus at its head and to the β4Thr site by hydrogen bonding with the hydrophilic functional group at its tail. The proponents of this model (Ross & Subramanian, 1977, 1978) have stated that, in general, "an effective inhibitor should contain both hydrophobic and hydrophilic groups (at specified distances) that match the original complementarity of the contact regions in the Hb S polymer". They further state that a more efficient inhibitor might be achieved by enhancing the polarizability of the aromatic nucleus by appropriate substitution with heavy halogen or aryl groups. That such is indeed the case has been amply demonstrated by the studies reported here which show, for example, that 5-bromotryptophan is roughly twice as effective as tryptophan and that (1-naphthyl)alanine is about 2.5 times more effective than phenylalanine (see values of slopes in Tables II and III, respectively). Our results, therefore, fully support this prediction of the model of Ross and Subramanian for the requirements of an effective noncovalent inhibitor of gelation. Within the context of the general structural formula for such compounds given earlier, our data indicate that for β-aryl-substituted alanines (in which $n = 2$), the most potent ϕ group was 7-azaindolyl, a heterocyclic analogue of the indolyl moiety of tryptophan. Furthermore, the profound enhancement in antigelling activity shown by 5-bromotryptophan relative to that of tryptophan suggests that a similar increase in potency should result from bromination at the 5 position of either the 7-azaindolyl or 3-benzothienyl ring. The antigelling capacity of such amphiphilic substances (i.e., hydrophobic at one end and hydrophilic at the other) could, in principle, be boosted even further by chemical modifications of the alanyl side chain yet to be explored.

Our data also reveal another aspect of the requirements for a maximally effective noncovalent inhibitor of gelation, heretofore unappreciated. That is, the hydrophilic end of the amphiphile should be both bifunctional and zwitterionic, as is the case for α-amino acids. Unfortunately, in the comprehensive survey of phenyl and related compounds carried out by Behe & Englander (1979), the pendant side chain of the aromatic moiety terminated in *either* a carboxyl or an amino group (i.e., these were monofunctional amphiphiles). We have shown, by contrast, that tryptophan, for example, is considerably more effective than either tryptamine or 3-indolepropionic acid (see Table III). It would seem, therefore, that yet another constraint can be imposed on the general structure of an effective noncovalent inhibitor, namely, that the presence of two functional groups of opposite charge ($-NH_3^+$ and $-COO^-$) at the polar terminus of the side chain results in a species that is considerably more potent than one which terminates in either functional group alone.[4] In light of the fact that the most potent agent to emerge from our survey (5-bromotryptophan) was nearly 5 times more effective than phenylalanine, to attempt to generalize, as Behe & Englander (1979) did, that the best noncovalent inhibitors of gelation are no better than the simplest phenyl compounds with regard to the level of potency required for clinical use is unduly pessimistic.

In conclusion, we feel that it may yet be possible to develop a noncovalent inhibitor from among the class of β-aryl-substituted alanines with sufficiently high antigelling capacity (roughly an order of magnitude greater than that of phenylalanine) to warrant its consideration for further in vitro trials as an antisickling agent. The principal requirements for an effective antisickling agent would be low toxicity and ready permeability to the red cell membrane. By suitable manipulation of either the hydrophobic or hydrophilic end (or both) of the amphiphilic compounds reported here, it should be possible to satisfy these requirements. One is cautiously optimistic, therefore, that some variation of these amphiphilic molecules will prove to be of potential therapeutic value in the treatment of sickle cell anemia.

[4] An apparent exception to this rule is the case of *N*-carbamoylphenylalanine, which was only marginally less effective than the unmodified parent compound.

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