15%. The molecular weight estimation obtained from 2-D slab electrophoresis might be subject to rather large errors for the low molecular weight protein species.

Isolation of small-subunit proteins from rat liver has been done by several workers [for a review, see Bielka & Stahl (1978)]. In particular, Wool and his co-workers reported the purification of almost all the small-subunit proteins (Collatz et al., 1976, 1977). However, there has been no report so far on the isolation of ribosomal proteins from lower eucaryotes such as yeast. The yeast proteins purified here do not represent a complete collection and consist of less than half of about 30 species in the small subunit. The remaining proteins could not be purified by the two-step chromatography described in this paper, and we are in the course of isolating them by other means.

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Quantitative Assessment of the Noncovalent Inhibition of Sickle Hemoglobin Gelation by Phenyl Derivatives and Other Known Agents†

Michael J. Behe and S. Walter Englander*

ABSTRACT: The ability of a variety of phenyl derivatives to inhibit sickle cell hemoglobin gelation was placed on a quantitative scale by parallel equilibrium and kinetic assays. Modifications of the phenyl ring studied include polar, nonpolar, and charged substituents, added aromatic rings, and loss of aromaticity. Other noncovalent inhibitors previously reported to have high potency were measured and placed on the same quantitative scale. Some phenyl derivatives were found to be as effective as any other known noncovalent antigelling agent. The phenyl compounds penetrate easily into red cells, and their potency is tolerant to chemical modification, which holds out the possibility of designing low-toxicity derivatives. On the negative side, the level of potency obtainable appears to be inadequate for clinical use. The best phenyl inhibitors display a functionally defined inhibitory constant (K_I) of 75 mM, and it can be estimated that inhibitor concentrations over 20 mM would be necessary to obtain minimal clinically significant benefit. Furthermore, with the variety of modifications tested here, no impressive increase in activity could be achieved over that found in the simplest phenyl compounds.

When sickle cell hemoglobin (HbS) is deoxygenated, it aggregates to form extended fibrillar structures (Murayama, 1966; Bertles et al., 1970). In the red cell these structures distort the membrane into bizarre shapes and rigidify the cell. The aggregation phenomenon can also be studied in concentrated solutions of HbS in which the result of self-association is the formation of semisolid gel. With other investigators, we reasoned that amino acids or small peptides might competitively inhibit specific contacts involved in the formation of HbS fibers. The literature contains reports of the inhibition of gelation by several amino acids and oligopeptides [see Dean & Schechter (1978)]. We tested the amino acids and found, in agreement with Noguchi & Schechter (1977, 1978), that only phenylalanine and tryptophan inhibit gelation at reasonable concentrations. Experiments with derivatives of these

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showed the inhibitory quality to be based mainly in the phenyl ring. A number of inhibitors containing aromatic rings have been found to inhibit HbS gelation. Inhibition has been demonstrated for phenylalanine and some simple aromatic molecules (Ross & Subramanian, 1977; Noguchi & Schechter, 1977, 1978), the natural product xanthoxylol extracted from an African tree root and a synthetic analogue thereof (Ekong et al., 1975; Poillon & Bertles, 1977), and some phenylalanine-containing peptides (Votano et al., 1977).

Here we report a systematic study of phenyl derivatives to evaluate the inhibitory role of hydrophobicity, polarity, charge, extension of the ring system, and synergistic effects of combined inhibitors. The phenyl derivatives and other previously reported noncovalent inhibitors were studied by parallel kinetic and equilibrium assays which allow all the inhibitors to be ranked on a quantitative scale so that they can be compared with each other and, more importantly, with the level of inhibitory potency required for clinical use. Even the best inhibitors appear to fall far short of this standard.

Materials and Methods

Blood was collected from donors known to be homozygous for HbS and to have negligible levels of fetal hemoglobin. Hemolysates were prepared by lysing the cells in distilled water and centrifuging out the cell membranes. The HbS thus obtained was dialyzed against 0.25 M potassium phosphate, pH 7.15, and concentrated to 34 g/dL in a collodion bag apparatus.

Compounds I, II, VII–X, XIII–XIX (Figure 1) and cyclohexylamine were purchased from the Aldrich Chemical Co. Compounds IV and V were purchased from Sigma. The phenylalanyl peptides XI and XII were the generous gift of Drs. J. Votano and A. Rich. All compounds were purchased in the best available grades and used without further purification.

The *N*-succinyl derivatives of phenethylamine and cyclohexylamine (compounds III and VI) were synthesized by the slow addition of 0.05 mol of the amine in 50 mL of acetone to 0.05 mol of succinic anhydride, also in 50 mL of acetone. The solutions were slowly cooled to −10 °C, where the products crystallized. Both derivatives were recrystallized from acetone.

To synthesize carbamylphenylalanine (compound XX), we added 1 g of KCNO to 2 g of phenylalanine in 10 mL of water at pH 10.0, the reaction mixture was kept at room temperature overnight and then acidified, and the precipitated product was removed by filtration. The product was recrystallized from ethanol.

To synthesize the active ester of *N*-succinylphenethylamine, we added 0.05 mol of dicyclohexylcarbodiimide in 10 mL of acetone to a solution of 0.05 mol of *N*-succinylphenethylamine and 0.05 mol of *N*-hydroxysuccinimide at room temperature in 30 mL of acetone. The mixture was incubated at room temperature overnight and the dicyclohexylurea filtered off.

For the covalent affinity modification of HbS, 100 μL of a 1.0 M solution of the active ester of *N*-succinylphenethylamine in acetone was added to 10 mL of 0.5 mM deoxy-HbS in 0.25 M potassium phosphate at pH 7.15. The solution was allowed to stand for 30 min at room temperature and then dialyzed against buffer. Disc gel electrophoresis showed an average of four modifications per hemoglobin molecule.

Equilibrium Assays. Assays were done to determine the equilibrium solubility of HbS in the presence of inhibitor (Bertles et al., 1970; Hofrichter et al., 1976). To 100 μL of concentrated HbS in 0.25 M phosphate, pH 7.15, was added 10 μL of a 0.75 M dithionite solution and 10 μL of a 0.6 M solution of inhibitor at pH 7. The final inhibitor concentration was 50 mM, and solution pH was 6.9. Solutions were placed in a 4-cm length of thin quartz tubing sealed at one end, incubated at 37 °C to achieve gelation, and reequilibrated in a 20 °C water bath for 20 min. They were then placed in glycerol-filled centrifuge tubes topped by a rubber ring to center the quartz tubing and centrifuged for 30 min at 150000*g* (40000 rpm in an SW60 Beckman rotor) with the temperature carefully maintained at 20 °C. The quartz tubing was broken 2 to 3 mm above the pelleted gel, 10 μL of the supernate was removed from the broken end, and the supernatant hemoglobin concentration was determined by absorbance at 540 nm after diluting by 300-fold into normally oxygenated buffer. No particular effort was necessary to exclude O_2 during the centrifugation; some O_2 can be seen to contaminate the upper millimeters of the open end of the tube, but this region is not sampled in the analysis. The equilibrium solubility assay uses a minimum amount of HbS (100 μL) and takes a short time (1 h), and many samples can be handled at the same time (we have studied up to 12 samples in one centrifugation). The assay provides a thermodynamically defined measurement of equilibrium HbS solubility (Magdoff-Fairchild et al., 1976). Similar equilibrium results for two of the phenyl derivatives we tested have been previously reported by Noguchi & Schechter (1977, 1978) and by Ross & Subramanian (1977) (see Table I).

Table I: Summary of Inhibition Results[a]

inhibitor	$[HbS]_{50}/[HbS]_0$ equilibrium assay	$[HbS]_{50}/[HbS]_0$ kinetic assay	t_{50}/t_0
butylurea[b]	1.21		450
XIII	1.21	1.20	400
DBA[c]	1.20		350
XII	1.19	1.20	300
VII	1.18	1.21	300
XI	1.17	1.20	250
XIV	1.17	1.20	250
I	1.15	1.17	120
I[d,e]	1.11; 1.19		100
Lys-Phe-Phe[e]	1.15		100
II	1.14	1.17	100
II[d,e]	1.18; 1.21		300
III	1.14	1.17	100
XVIII	1.15	1.16	100
VIII	1.12	1.14	50
X	1.12	1.14	50
IX	1.11	1.14	40
XX	1.12	1.11	30
IV	1.07	1.08	10
V	1.05	1.08	10
VI	1.06	1.08	10
XV	1.06	1.06	5
XVI	1.06	1.06	5
XIX	1.05	1.06	5
XVII	1.03	1.04	3
ethanol[c,d]	1.01; 1.05		1
pro-Val	1.00		1
retinol	1.00		1
lysolecithin	1.00		1

[a] The equilibrium assay (column 2) measures the ratio of solubilities of HbS in the presence and absence of 50 mM inhibitor. The kinetic assay (column 3) measures the ratio of concentrations needed to maintain a constant delay time in the presence and absence of 50 mM inhibitor. The delay time ratio (column 4) was calculated as the 32nd power of the concentration ratio. Literature values referenced were recalculated to the present basis. [b] Elbaum et al. (1974). [c] Poillon & Bertles (1977). DBA is 3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-6-butyric acid. [d] Ross & Subramanian (1977). [e] Noguchi & Schechter (1977).

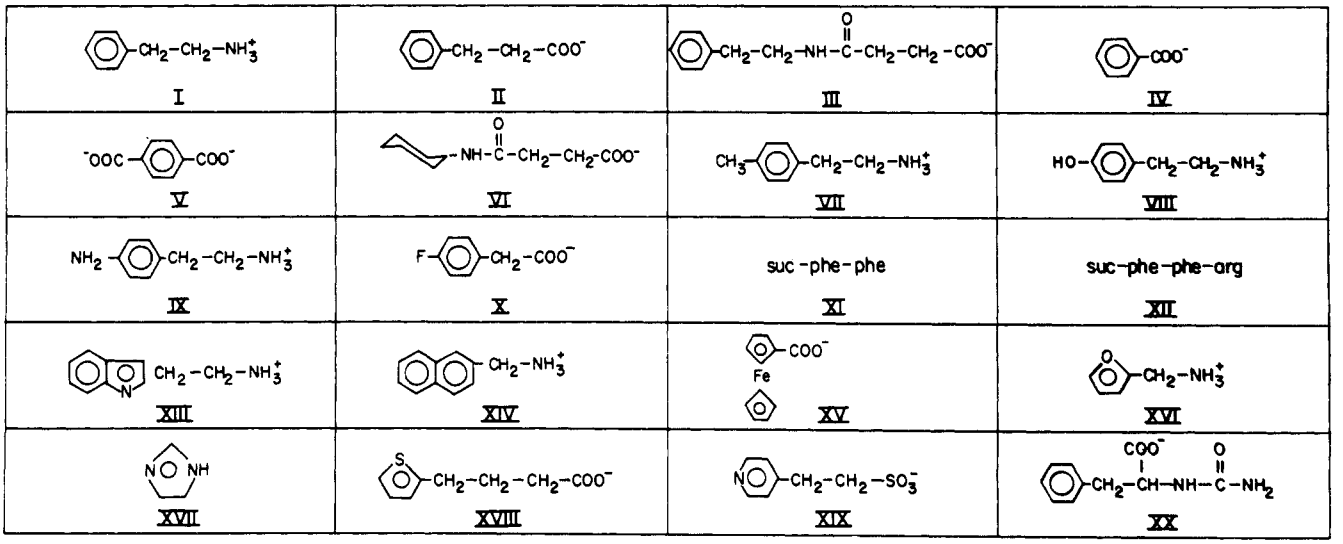

FIGURE 1: Compounds tested by our assay systems: (I) phenethylamine; (II) hydrocinnamic acid; (III) *N*-succinylphenethylamine; (IV) benzoic acid; (V) terephthalic acid; (VI) *N*-succinylcyclohexylamine; (VII) *p*-toluylethylamine; (VIII) tyramine; (IX) *p*-aminophenethylamine; (X) *p*-fluorophenylacetic acid; (XI) *N*-succinylphenylalanylphenylalanine; (XII) *N*-succinylphenylalanylphenylalanylarginine; (XIII) tryptamine; (XIV) β-naphthylmethylamine; (XV) ferrocenecarboxylic acid; (XVI) furfurylamine; (XVII) imidazole; (XVIII) thiophenebutyric acid; (XIX) pyridineethanesulfonic acid; (XX) *N*-carbamylphenylalanine.

Kinetic Assays. Upon deoxygenation HbS solutions display a latent period (delay time) in which no changes in solution properties are evident, followed by a rapid increase in viscosity to form the gel. Hofrichter et al. (1974) interpreted this in terms of a rate-determining nucleation step. In our hands, the delay time is dependent on the ~32nd power of the HbS concentration in the concentration range around 20 g/dL (Behe & Englander, 1978).

Kinetic assays of inhibitory potency may be performed in various ways. Most simply, delay time can be measured as a function of inhibitor concentration. We have found (Behe & Englander, 1978) that greater accuracy and convenience can be achieved by arranging the assay to maintain a constant delay time while covarying inhibitor and HbS concentration. A further benefit for present purposes is that results of the equilibrium and kinetic assays then appear in directly comparable terms. In both assays the inhibitor can be pictured as "inactivating" a fraction of the HbS present so that, at any given inhibitor concentration, it is necessary to increase HbS concentration in order to regain the initial equilibrium gelling activity or the initial kinetic gelling rate.

The kinetic assay determines the increased concentration of HbS necessary to recover a preset delay time in the presence of increasing concentration of inhibitor. To 100 μL of concentrated HbS in a small test tube at 0 °C was added 10 μL of 0.75 M dithionite and 10 μL of a concentrated solution of inhibitor which had been titrated to neutrality. The final pH was 6.9. The solution was then repeatedly gelled under argon at 37 °C, reliquified in an ice bath, diluted by judicious addition of buffer, and gelled again until the delay time reached 150 ± 20 s. At this point the HbS and inhibitor concentrations were recorded. This procedure requires about 10 min. Because the dependence of the delay time on concentrations is so large, the uncertainty of 15% in delay time corresponds to an uncertainty of 0.5% in HbS concentration and thus does not limit experimental accuracy. Temperature reequilibration from 0 to 37 °C requires about 30 s, which corresponds to an integrated equivalent of 5 s at 37 °C as far as the gelling process is concerned. The delay time end point was determined simply by observing failure of the solution to flow when the test tube was tilted. With this method the solutions are subjected to no significant shearing force.

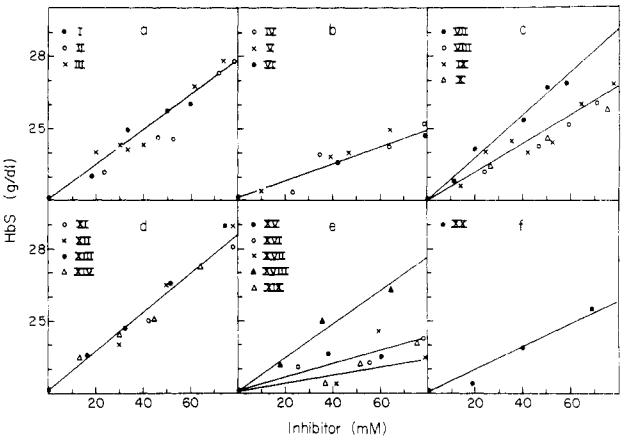

FIGURE 2: Kinetic assays of inhibitory potency. The curves represent the increasing concentration of HbS necessary to maintain a constant delay time (150 s at 37 °C) as inhibitor concentration is increased.

Results

Kinetic and Equilibrium Assays. The phenyl derivatives and analogues studied are shown in Figure 1. Figure 2 shows the results of kinetic assays which determine the increased concentration of HbS necessary to recover a constant delay time (150 s at 37 °C) in the presence of increasing concentrations of inhibitor. Table I summarizes the kinetic assays in terms of the ratio of those concentrations of HbS which yield a constant delay time in the presence and absence of 50 mM inhibitor. Also shown in Table I are the results of analogous equilibrium assays in which the concentration of sol phase HbS in equilibrium with its gel was measured in the presence and absence of 50 mM inhibitor. The kinetic and the equilibrium assays provide independent but comparable estimates of the ability of various compounds to reduce the gelling activity of HbS, and these two measures are in good agreement over the entire scale (Table I).

The concentration ratios obtained from both assays can be converted to the change in the gelation delay time brought about by the addition of 50 mM inhibitor. This computation uses a modified form of the supersaturation equation (Hofrichter et al., 1974). That is, in the absence of inhibitor these solutions would differ in delay time by the factor t_{50}/t_0 =

$([HbS]_{50}/[HbS]_0)^{32}$. With 50 mM inhibitor added to the more concentrated HbS solution, delay times are made the same. Thus, addition of the inhibitor slows gelation by the factor $([HbS]_{50}/[HbS]_0)^{32}$. Table I lists delay time ratios calculated in this way from the average of the kinetic and equilibrium results.

The delay time ratio provides a physically meaningful quantitative measure of inhibitor potency. The effect of added inhibitor on both the kinetic and equilibrium parameters can be understood in terms of the "inactivation" by the inhibitor of a fraction of the HbS. Additionally, this value has been given direct clinical relevance by the analysis of Sunshine et al. (1978) which scales the relative delay time to clinical severity of the disease. We will return to these issues under Discussion.

Phenyl Derivatives. The focus of this communication is the overall level of inhibitory power attained by the class of compounds studied here. Nevertheless, the detailed effects of various chemical groupings have formed the basis of a number of earlier studies and the compounds studied here may contribute to this ongoing discussion.

The monosubstituted phenyl derivatives I–III, which vary in the sign of their charge and its distance from the phenyl ring, give similar inhibition of HbS gelation (Figure 2a). Evidently, inhibitor activity is not very sensitive to changes in the charge and length of the "tail" of the molecule. However, a charged group next to the ring as in IV and V or the loss of aromaticity as in VI greatly reduces the inhibitory effect (Figure 2b). Evidently, the aromatic character of the ring is important but not in itself sufficient for inhibition [see also Noguchi & Schechter (1978)].

The effect of other substitutions are shown in Figure 2c. The addition of a hydrophobic methyl group slightly increases the inhibitory potency of VII compared to the monosubstituted compounds I–III. The addition of a polar OH, NH_2, or F group to the ring slightly decreases the inhibitory potency (VIII, IX, and X). However, the uncharged polar groups do not weaken the inhibition nearly as much as the full charge in IV and V.

Figure 2d shows the effect of aromatic molecules with extended ring systems. Both XIII and XIV, which have two fused rings, are only slightly better inhibitors than the single-ring compounds I–III. Figure 2d also measures the activity of oligopeptides (XI and XII) which contain two phenyl groups. Noguchi & Schechter (1978) found an identical result for a similar peptide, Lys-Phe-Phe (Table I, recalculated to the present basis). The peptides are, on a molar basis, slightly better inhibitors of gelation than I–III, but are less effective inhibitors if the molarity of phenyl groups is considered.

The effect on inhibition of small aromatic rings and heteroatoms was investigated (Figure 2e). XV, which possesses an aromatic cyclopentadienyl ring, has low activity, comparable to the nonaromatic VI. Although VIII and IX, with uncharged polar atoms attached to the aromatic ring, are effective inhibitors, XVI and XVII, with polar atoms as part of their aromatic ring, are relatively poor inhibitors. XVII, which has two heteroatoms, is the weakest of the inhibitors. The six-membered pyridine ring derivative XIX is also seen to be a poor inhibitor of gelation. XVIII, which has a relatively nonpolar sulfur as its heteroatom, is as effective as I–III.

It has been reported that substituted ureas are effective inhibitors of HbS gelation (Elbaum et al., 1974). Consequently, we synthesized XX, which combines both a substituted urea and a phenyl group, hoping to find a synergistic effect. As shown in Figure 2f, XX did not prove to be more effective than simple phenyl compounds.

Nonphenyl Inhibitors. Elbaum et al. (1974) have investigated the effect of alkylureas on the minimum gelling concentration of HbS and found butylurea to be most effective of the compounds they tested. Table I shows that butylurea is about as effective as the best phenyl inhibitors of gelation. Several workers have quantified the effect of ethanol on the solubility of HbS. Table I ranks ethanol as a weak inhibitor. Other organic solvents (dimethyl sulfoxide, dimethylformamide, and dioxane) were tested by Waterman et al. (1974) for their effect on HbS solubility. All the organic solvents were shown by these investigators to be about as effective as ethanol in solubilizing HbS. Several amino acids besides phenylalanine have been reported to inhibit gelation (Sophianopoulis et al., 1974; Rumen, 1975). However, in agreement with Noguchi & Schechter (1977, 1978), we find that no amino acids other than phenylalanine and tryptophan inhibit gelation significantly. Several gases such as carbon monoxide and dichloromethane (Schoenborn, 1976) inhibit gelation by binding to HbS, but these are too toxic for maintenance therapy.

Other Reportedly Effective Inhibitors. The literature records three studies of substances that appeared to inhibit HbS gelation extremely effectively.

A class of peptides containing phenylalanine was reported by Votano et al. (1977) to inhibit gelation more potently than phenylalanine itself. The presence of 70 mM peptide inhibitor was reported to increase the delay time of HbS gelation from under 2 min to longer than the 30-min cutoff time of these experiments. Two of these peptides, XI and XII, when tested by our kinetic assay, do display good inhibitory power (Table I). However, the peptides do not totally inhibit gelation as reported. The peptides have a t_{50}/t_0 value of 250–300 on a mole of peptide basis, which is similar to the monophenyl compounds, though this value falls to 20–25 when the molarity of phenyl groups is considered.

Other oligopeptide inhibitors have been studied by Kubota & Yang (1977), who used an assay similar to the minimum gelling concentration (MGC) test of Bookchin et al. (1970). In this assay, deoxy-HbS solution is concentrated by evaporation in a stream of dry gas until gelation occurs and the HbS concentration at this point defines the MGC value. Kubota & Yang (1977) found the apparent MGC of HbS to be increased by every one of a variety of oligopeptides they tested. Oligopeptides used included fragments of HbS and HbA, analogous scrambled-sequence fragments, Leu- and Met-enkephalen, and $(Pro)_6$. One of these peptides, Pro-Val, the dipeptide at the HbS mutation site, when tested by our kinetic assay at the highest concentration used by Kubota & Yang and at 4 times higher concentration, had no measurable effect (Table I). It may be pertinent that the MGC measured by these investigators for HbS alone is much lower than the values published by others and that under the most highly "inhibited" conditions used the MGC rose to values reported by others for HbS alone.

An exceptional degree of HbS gelation inhibition has been reported for certain agents by Freedman et al. (1973), who also used an MGC assay. Lysolecithin and retinol appeared to be highly effective at a concentration of only 1 mM, but large effects were recorded also for some simple sugars. When tested by our kinetic assay even at the higher concentration of 8 mM, a limit dictated by the relative insolubility of these compounds, lysolecithin and retinol were without effect (Table I).

In light of these comparisons, it appears that the phenyl derivatives at the top of the list in Table I provide, together

with butylurea, the most potent noncovalent inhibitors of HbS gelation so far available.

Covalent Labeling of HbS. A problem with any noncovalent inhibitor of HbS gelation in vivo would be its progressive removal from the serum. On the other hand, if a high steady-state level of free inhibitor is maintained, then toxic side effects seem likely. Both problems might be overcome if the inhibitory group, while noncovalently bound at its inhibitory sites, could be covalently attached to a nearby HbS side chain. This tactic might also provide a large effective concentration of inhibitor in the vicinity of the binding site.

To explore this possibility, we synthesized the *N*-hydroxysuccinimide active ester of III. Active esters can react with the abundant lysines in HbS to give the corresponding amides. Deoxy-HbS was reacted with the ester until an average of four lysines per tetramer were modified (monitored by disc gel electrophoresis). (Reaction with oxy-Hb inhibited the allosteric transition to the deoxy form.) Neither the kinetic nor the equilibrium gelling assay showed any difference from unmodified HbS.

Red Cell Permeation. In addition to their ability to inhibit HbS gelation, the small molecule phenyl derivatives penetrate easily through the red cell membrane. We observed this by placing inhibitors in solution with red cells at a high volume concentration and centrifuging down the red cells. The movement of phenyl compounds I–III into the red cells was measured as a loss in spectral absorbance of the compound in the supernate.

In these experiments a centrifuged red cell pellet was resuspended in an equal volume of buffer containing 10 mM inhibitor, and after a brief equilibration period the red cells were again pelletted. The absorbance remaining in the supernate due to the phenyl derivative quickly decreased to 60% of the initial value. This is the expected decrease if the phenyl compounds fully equilibrate with the internal red cell space. The possibility that the observed loss represents dissolution into the red cell membrane seems unlikely in view of the high intramembrane concentration that would be required. Further, if it is admitted that these water-soluble compounds can penetrate into the membrane, then they must perforce equilibrate with the intracellular aqueous space.

Compounds I and II reached the equilibrium level within 10 min. Compound III was slower and required 20 min for equilibration.

Strength of Binding. A functional inhibitory constant (K_I) for the inhibitors studied can be obtained from the results in Figure 2 by plotting the fraction of HbS inactivated against inhibitor concentration. The defining function for both the kinetic and equilibrium results measured in the kinds of experiments performed here is given in eq 1 [see Behe & Englander (1979)].

$$K = \gamma_0[\mathrm{HbS}]_0 = \gamma_I f_I[\mathrm{HbS}]_I \qquad (1)$$

The fraction of HbS still active in the presence of inhibitor (f_I) can be calculated for each data point in Figure 2 as follows:

$$f_I = \gamma_0[\mathrm{HbS}]_0/\gamma_I[\mathrm{HbS}]_I \qquad (2)$$

Here K represents the gelling activity as defined by Behe & Englander (1979); γ_0 and γ_I are activity coefficients for the solutions without and with inhibitor, respectively. Equation 1 expresses the fact that in a sol–gel equilibrium system solubility in the sol phase, expressed in terms of protein activity, γ[HbS], is constant and that when a fraction of the HbS present is inactivated by added inhibitor the parameter that is maintained constant in the equilibrium sol is the gelling activity, γf[HbS]. Kinetic experiments performed as in this work, with the delay time kept constant, provide an independent method for experimentally defining constant gelling activity (eq 1) and thus determining the fraction of HbS inactivated by added inhibitor.

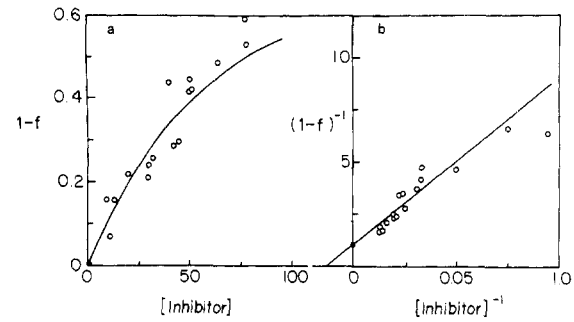

FIGURE 3: (a) Inhibition curve plotted as the fraction of HbS inactive for gelation ($1 - f$) vs. the concentration of inhibitors VII and XI–XIV. Data were calculated according to eq 2 from the kinetic results in Figure 2. The curve drawn represents the straight line in part b. (b) Inverse plot of the data in part a. Independently of any binding model, this curve is constrained to pass through the point plotted at unity on the ordinate.

The fraction of HbS inactivated, $1 - f$, is plotted in Figure 3a against inhibitor concentration by using the data in Figure 2 for the five best phenyl inhibitors (VII, XI–XIV), which essentially superimpose. Figure 3b is the corresponding inverse plot. In calculating f (eq 2), we took values for $\gamma_I/\gamma_0 = \gamma_r$ as a function of total hemoglobin concentration from Behe & Englander (1979). A functional K_I of 75 mM is indicated by these results. Evidently, the binding that inhibits gelation displays very low affinity.

This treatment makes no assumptions about the number of phenyl-binding sites or their relative efficiency in inhibiting gelation. Ross & Subramanian (1977) have suggested that binding at a single site fully inactivates the HbS molecule. The present data are consistent with this interpretation but are not sufficient to rule out other more complex possibilities. The data do clearly rule out any high-affinity inhibitory binding and provide a K_I value which can be used to infer in vivo effects on delay time, and therefore on clinical efficacy (Sunshine et al., 1978), at levels of inhibitor well below the 75-mM level (see Discussion).

Discussion

The results obtained here allow quantitative comparisons to be made among various members of the phenyl class of gelation inhibitors and with other noncovalent inhibitors of gelation previously reported. On this relative scale, some phenyl derivatives rank as high as any other known inhibitor. The results also allow one to rank these inhibitors on an absolute scale, namely, in terms of the concentration necessary to achieve a significant clinical effect.

How effective would the best phenyl derivatives be in alleviating sickle cell disease? Sunshine et al. (1978) have recently observed that a 10- to 100-fold increase in the delay time of gelation can be correlated with a somewhat milder clinical course; to achieve great benefit, factors of 10^3–10^4 are necessary. Let us calculate the approximate concentration of phenyl inhibitor necessary to increase delay time by a factor of 10. We have previously shown (Behe & Englander, 1978) that at constant protein concentration the delay time of HbS gelation is inversely proportional to the 10th power of "active" HbS. The 10th power holds when, as in our experiments and as would be true in vivo, HbS concentration is held constant and some fraction is rendered unable to participate in gelation. To obtain a 10-fold slowing in gelation rate then while holding

total HbS concentration constant, ~20% of the HbS must be inactivated, since $(1.0 - 0.2)^{10} = 10^{-1}$. A 100-fold slowing requires the inactivation of 37% of the HbS. As indicated above, the functional K_I for the best phenyl inhibitors is ~75 mM. Thus, a 20–40 mM concentration of inhibitor would be necessary for minimal clinical benefit. Since these reagents are membrane permeable, one must contemplate a drug dosage in the 100–200-g range to bring total body water to this level.

Can this extraordinary concentration of administered drug be biologically acceptable on a continuing basis? The tolerance of the inhibitory potency of the phenyl compounds to chemical modification may be considered promising in this regard since this presents the possibility of designing low-toxicity derivatives. On the other hand, it seems likely that at least one order of magnitude increase in inhibitory power and probably more will still be necessary. In this sense, the difficulty of greatly improving the inhibitory power experienced in this work seems a pessimistic indication.

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