# Rational Approaches to Chemotherapy: Antisickling Agents

Irving M. Klotz, David N. Haney, L. Carroll King

*Summary.* The rationale leading to the design and synthesis of double-headed aspirins as potential antisickling agents is described.

Biomedical scientists with a chemical orientation recognize that the rational design of therapeutic compounds must be based on an understanding of a disease at a molecular level; that is, biological function is embedded in molecular structure. A major obstacle to success in the rational design of specific drugs is our ignorance of the molecular basis of disease. However, there is one notable exception to this ignorance: namely, sickle cell disease. After decades of research, it is now known that the pathophysiology of the sickle cell syndrome is the consequence of an aggregation process arising from a specific substitution of a single amino acid residue in the β chains of hemoglobin.

Sickle cell anemia was first recognized in 1905 by J. B. Herrick (*1*), a cardiologist and professor at Rush Medical College and attending physician at Presbyterian Hospital in Chicago. It was he who discovered peculiar sickle-shaped erythrocytes in a sample of blood from an anemic West Indian clinical patient (Fig. 1). The first indication that sickle cell hemoglobin (HbS) differs in a molecular structural feature from normal adult hemoglobin (HbA) was provided by Pauling, Itano, Singer, and Wells (*2*) almost 40 years later. These investigators found that in the 64,000 molecular weight hemoglobin molecule there exists a small, but definite, difference in electrical charge between HbS and HbA. Subsequently Ingram (*3*) showed that this difference is due to the replacement of a single glutamic acid residue by a valine in one of the two types of chain in hemoglobin. Hemoglobin consists of two identical α and two identical β chains, each containing 141 and 146 amino acid residues (*4*), respectively. A single residue mutation in the sixth position in the β chains

HbA: Val-His-Leu-Thr-Pro-*Glu*-Glu-Lys-· · ·

HbS: Val-His-Leu-Thr-Pro-*Val*-Glu-Lys-· · ·

leads to an aggregation of HbS molecules into long fibers (Fig. 2) which distort the erythrocyte into the characteristic deformed shape associated with sickle cell syndrome (*5*).

The precise structural arrangement of hemoglobin molecules within these fibers has yet to be established unequivocally. Nevertheless, electron microscopy and image reconstructions (*5–16*) combined with information obtained from x-ray diffraction measurements (*11, 17–20*) are beginning to reveal the orientations of individual macromolecules within the filaments that constitute a fiber. At present, it seems likely that a 14-strand arrangement (ten outer filaments surrounding four inner ones) occurs in the majority of the aggregated HbS molecules in a sickled erythrocyte.

Points of contact between hemoglobin molecules in the fiber have not yet been delineated with certitude, but educated guesses have been made especially from electron microscope and x-ray studies of fibers and crystals (*19, 21, 22*) and from comparisons of sickling tendencies of hemoglobin double mutants (*23–31*). There are strong indications that the surface contacts of HbS tetramers in fibers involve the same residues as in crystals, where a hydrophobic bond is formed between β6 Val of one hemoglobin tetramer and β85 Phe and β88 Leu of an adjacent tetramer. This interaction seems to be crucial, but there are also strong intimations of a large number of other contacts involving electrostatic cross-links, hydrogen bonds, and nonpolar interactions. The specific residues implicated in contacts (Fig. 3) show the partners involved. All of these seem to play a role in influencing the solubility and consequent polymerization of sickle hemoglobin.

With this molecular information, we can devise a variety of strategies for counteracting sickling. One general scheme involves perturbing HbS at sites in the contact interfaces of the protein molecules on the insoluble fiber so that intermolecular interactions are obstructed directly. Alternatively, an indirect, flanking strategy can also be envisaged, based on the following. More than 50 years ago, Hahn and Gillespie (*32*) found that sickling depended on lower oxygen tension; that is, on the formation of deoxyhemoglobin. Subsequently, Muirhead and Perutz (*33, 34*) showed that the geometry of the tetrameric form of deoxyhemoglobin is substantially different from that of the oxy form. For HbS it is the deoxy tetramer that assembles in the fibrous gel. The deoxy quaternary conformation has the spatial arrangement of surface groups that are in register to link the tetramers together to form an extended fibril. In contrast, in the oxy conformation, corresponding interactions do not exist. If we could shift the conformational equilibrium toward the oxy structure, or better yet if we could covalently lock contact regions in a conformation out of register for aggregation, sickling tendencies should be reduced.

In actual practice, a large number of compounds have been examined, without clear molecular rationalization, for antisickling potential. Many compounds were tried before even any hints were available as to the molecular nature of contact interfaces, and even now with some elucidation of this structural question, no novel specific chemical or physicochemical reaction for approaching the aggregation problem directly has emerged. In reviewing the reagents tried, therefore, we categorize them at first in terms of their chemistry as noncovalent or covalent in their mechanism of action.

## Noncovalent Agents

For several decades, it was "well known" that urea (**1**) breaks hydrogen bonds. Since even in the 1950's hydrogen bonds were recognized to play a role in protein folding and aggregation, Alli-

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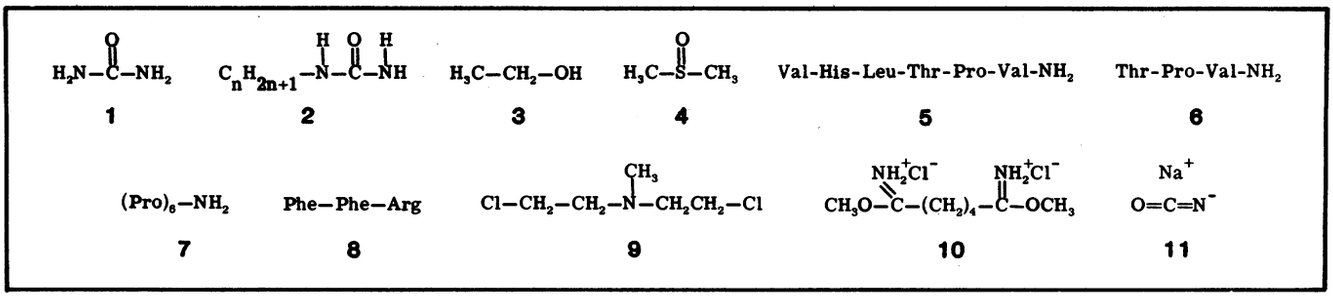



son (*35*) added urea and found that HbS aggregation could indeed be diminished. Subsequently, after the action of urea on proteins was "well-known" to be due to interference with hydrophobic bonds, Nalbandian (*36*) proposed and used it for the clinical treatment of sickle disease and reported favorable results, but these were not confirmed in tests with suitable controls. Regardless of the theoretical premise for its use, in practice urea affects proteins only when its concentration is high (approximately molar) and hence has never really been a promising candidate for clinical use. Alternatively, substituted alkylureas (**2**) have also been tried (*37*), but they too are effective only at relatively high concentrations (about 0.1*M*).

Another agent that interferes with nonpolar interactions in proteins and at a much lower concentration than does urea is ethanol (**3**), so it would be expected to diminish HbS aggregation (*38*). Indeed, 0.17*M* ethanol has been shown to decrease sickling substantially (*39, 40*). This concentration corresponds to about 700 milligrams per 100 milliliters of blood, which is near a fatal dose; but at half that concentration, only a high euphoria would be produced. A number of other polar organic solvents (*39*), such as dimethyl sulfoxide (**4**), dioxane, and dimethyl formamide also increase the solubility of HbS about as effectively as ethanol, but none seems an attractive candidate for therapeutic use.

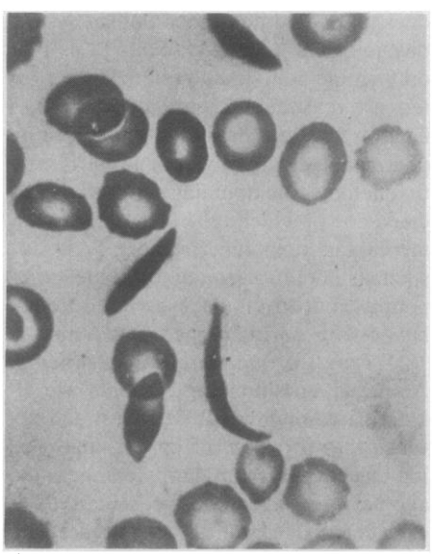


Fig. 1. Reproduction of microphotograph taken from the original publication of Herrick (*1*) showing "the peculiar elongated forms of the red corpuscles" of a person with sickle cell anemia.

Another rationale has led to the examination of a very different class of organic compounds. For many years it has been recognized that protein molecules in specific aggregates must have complementary polypeptide interfaces. It follows then that smaller oligopeptides with sequences mimicking those of one of the constituents at such an interface might compete with one of the protein macromolecule partners at the junction, and hence disrupt the aggregate. In regard to HbS aggregates, Yang and co-workers (*41, 42*) proposed that small peptides with structures similar to those in the region of the β6 Val of HbS should inhibit aggregation. Increases in minimum gelling concentration were indeed observed for peptides **5** and **6**, but also for **7** and for Leu- and Met-enkephalin (which are brain pentapeptides unrelated to **5**, **6**, and **7**). Subsequently, a variety of even smaller unrelated peptides, such as **8**, were also found to inhibit gelation of HbS (*43, 44*), and ultimately even simpler phenyl derivatives of a wide range of organic compounds (*45, 46*) were discovered to be effective. In general, it appears that compounds with apolar substituents can inhibit aggregation. That the mechanism is a highly specific structural displacement at contact interfaces seems unlikely, however. It has been known for some time that benzenoid and alicyclic compounds are bound by hemoglobin, as is evident, for example, from studies of crystallization of this oxygen carrier in the presence of such compounds (*47*).

Furthermore, a vast selection of organic anions of widely differing structure can be bound in the β cleft of hemoglobin. In addition to the naturally occurring modulators 2,3,-diphosphoglycerate (*48–50*) and inositol hexaphosphate, a range of organic (and inorganic) anions are capable of lodging in the β cleft (*51*) as is apparent from their effects on $O_2$ affinity of hemoglobin. Included in these compounds are even cellular metabolites, such as succinate and malate (*51*), and probably substances such as DBA (3,4-dihydro-2,2-dimethyl-2*H*-1-benzopyran-6-butyric acid) (*40*), which are analogs of natural products.

In many cases, such as those of DBA and dianions, the compounds cannot penetrate the erythrocyte membrane (*52*)

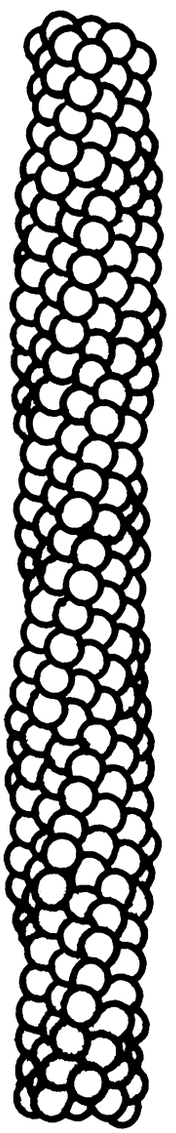
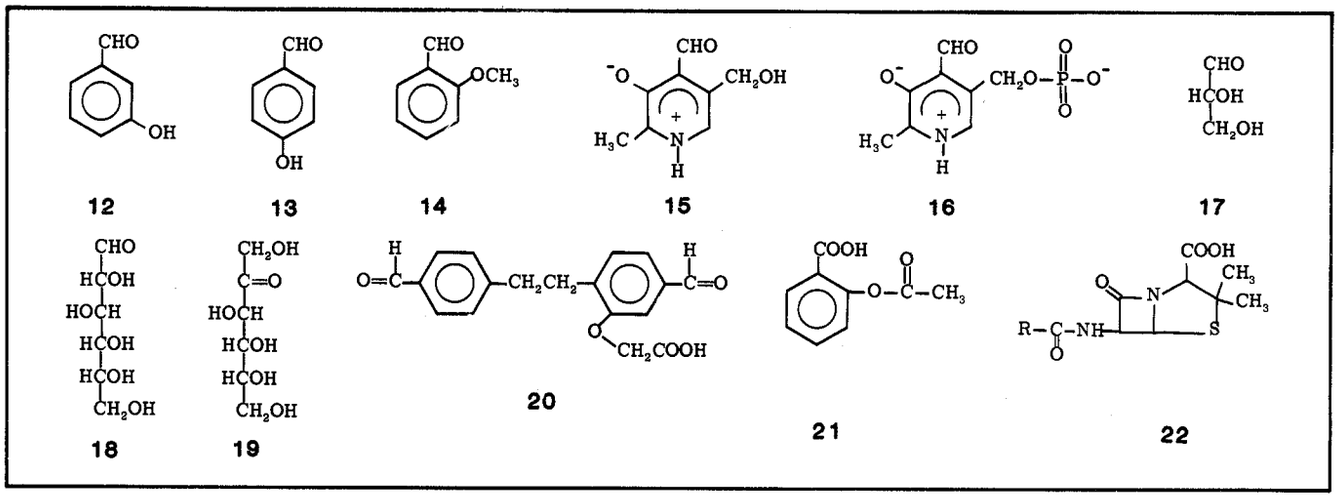


Fig. 2. Proposed arrangement of tetramers of hemoglobin along axis in sickle fiber. [Adapted from Dykes *et al.* (*14*)]




and hence will not be effective in vivo. In contrast, polarizable groups, such as Br substituents on aromatic rings, endow otherwise nonpermeating compounds with the ability to penetrate the red cell membrane (*53*) and also to increase their binding by hemoglobin. It may be, therefore, that the variations in severity in clinical manifestations of sickle cell disease are reflections of differences in the presence of small amounts of unrecognized dietary or metabolic products within the erythrocyte. Even if they only affected oxygen affinity, they could influence the onset of sickling. If they also were bound at contact interfaces or modified the conformation of hemoglobin, they could exert more direct effects on aggregation.

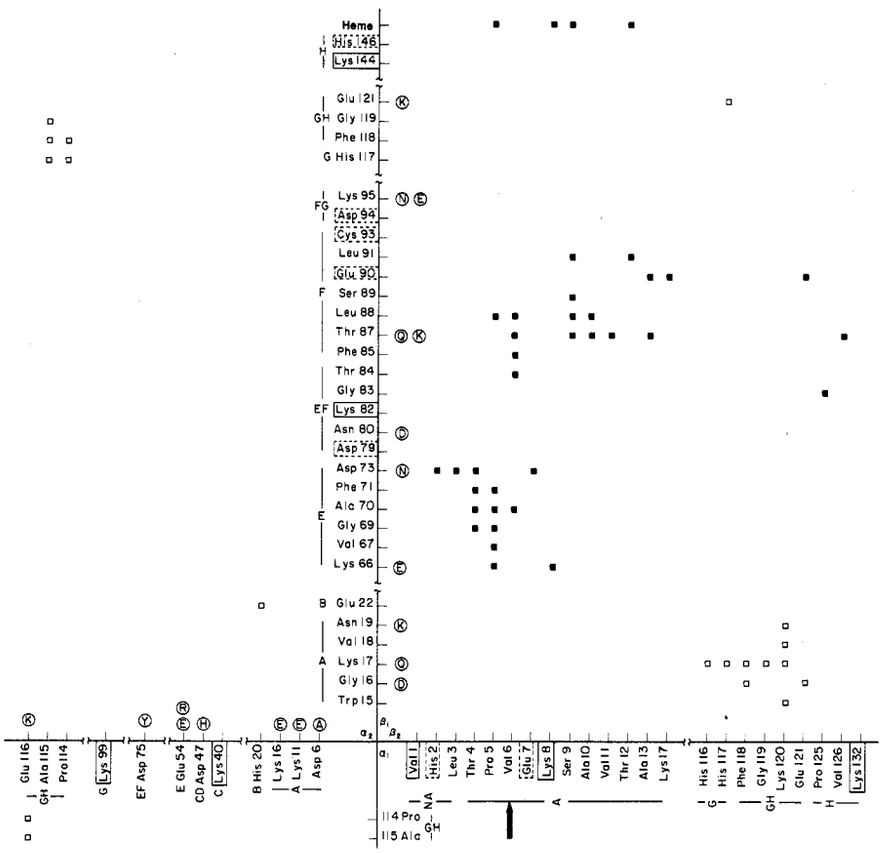


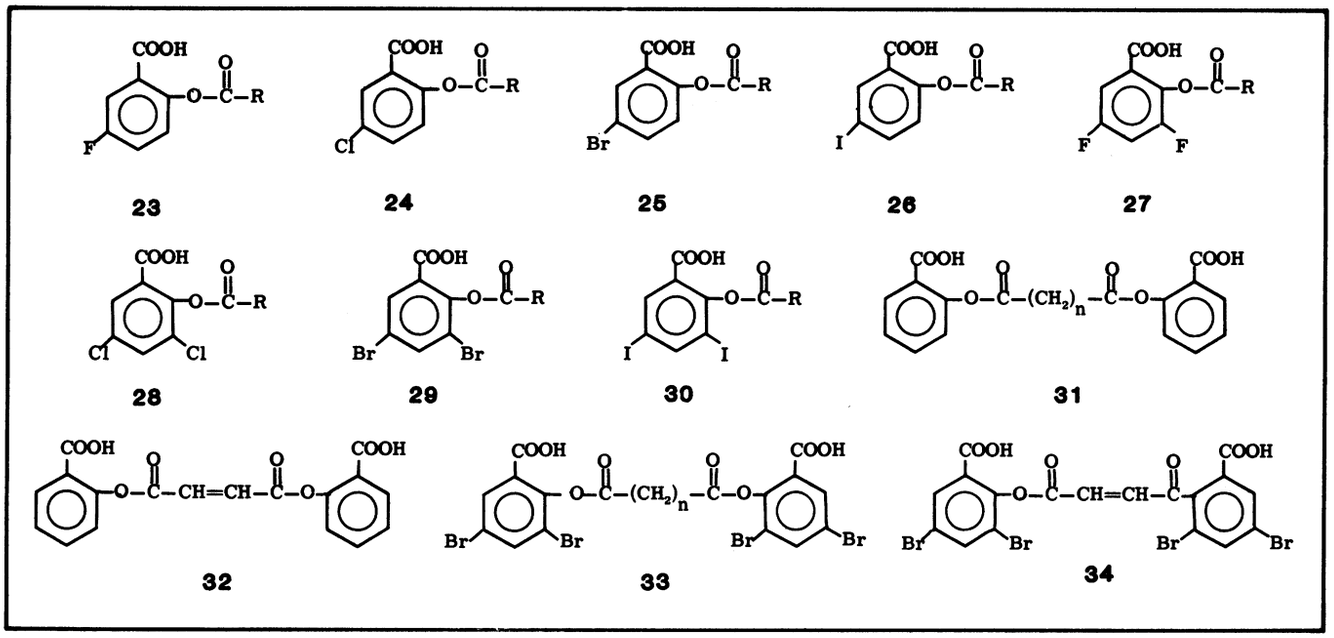

Fig. 3. Amino acid residues proposed to be in intermolecular contact regions between hemoglobin molecules in sickle fiber. The specific residues shown are suggested mainly from x-ray studies of deoxy HbS (*19, 21, 22*) and from comparisons of sickling tendencies of hemoglobin double mutants (*23—31*). □, Residues in up-down contacts along fiber axis; ■, residues in regions of side-to-side contacts. Circled letters designate mutants with single amino acid substitutions that alter gelation behavior. Amino acids boxed within a solid-line rectangle *have primary amine* groups available for acylation and are within the β cleft. Amino acids within a broken-line rectangle are potential nucleophiles that might be alkylated and are near the same region. The subscripts of the subunits, $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, denote binding site of type 1 or type 2 for chains on adjacent molecules. Also shown along the axes are portions of the eight helical regions (*A*, . . . *H*) and the six nonhelical regions (*NA*, *EF*. . . .) of the hemoglobin molecule.

### Covalent Agents

If covalent modification is to be used, two different molecular rationales can be envisaged. The most obvious is to attempt to modify HbS at sites in the contact interfaces of the protein molecules in the insoluble fibril so that intermolecular interactions are obstructed directly. Specifically, this approach suggests that β6 Val, β85 Phe, or β88 Leu be altered. However, we have been unable to find a record of any reaction for tampering with these hydrophobic residues that could be carried out safely in a physiological milieu. It seems to us that only amine-containing residues in a protein are reasonable candidates for modification with reagents mild enough to be pharmacologically acceptable. It is true that hemoglobin contains sulfhydryl residues, one of which, β93 Cys, reacts with mercaptan-blocking reagents (*35, 54, 55*) such as mercurials and disulfide compounds, but in vivo these would wreak havoc with a wide range of enzymes.

1) *Types of amine-blocking agents.* Among amine-blocking reagents are alkylating compounds, aldehydes, and ketones that form Schiff bases, amidinating reagents, carbamylating compounds, and acylating agents. Representatives of each class have been shown to react with hemoglobin in vitro.

An exceptionally effective alkylating agent for hemoglobin is nitrogen mustard (**9**), which markedly inhibits sickling (*56*) in vitro. Although this compound may be




useful in extracorporeal therapy, it is obviously not suitable for oral or intravenous treatment.

The interactions of carbonyl compounds with amines of hemoglobin have been examined on a very broad scale (for example, **12** to **20**). In vivo, the appearance of $HbA_{Ic}$, $HbA_{Ia2}$, and $HbA_{Ia1}$, adducts of hemoglobin with glucose, glucose 6-phosphate, and fructose 1,6-diphosphate, respectively (*57–59*), in the blood of normal individuals points strongly to Schiff base formation between carbohydrates and the β chains of this oxygen carrier under physiological conditions. That such sugar adducts are formed in vitro has been demonstrated with hemoglobin (*59–61*) as well as with a Val-His peptide (*62*) corresponding to the β amino terminus. Such observations suggested the use of glyceraldehyde (*63, 64*), since it is an open chain, as an antisickling agent, but it is apparent that the concentrations required are too high. In a separate direction, there have been extensive studies (*65–67*) with aldehydic pyridoxal derivatives (see **15** for base structure) which show some specificity for the β-cleft region. Beddell *et al.* (*68, 69*) have also designed a di-aldehyde, 4,4′-diformyl-2-bibenzyloxyacetic acid (**20**), with a structure that fits well in the β cleft. Although Schiff base formation is a reversible reaction, compounds of this class may prove to be effective antisickling agents if they are bound strongly and specifically enough at low concentrations.

Imidates are also reactive toward amine groups and hence are effective modifiers of Lys residues in proteins. Dimethyladipimidate (**10**) has been particularly popular in cross-linking reactions with proteins, but the reaction has to be carried out at a *p*H substantially above 8. Dimethyladipimidate reacts with the erythrocyte membrane (*70*) as well as with hemoglobin (*71*), as does the monofunctional methylacetimidate (*72*).

In the early 1970's when urea was thought to be effective clinically at relatively low concentrations and yet was known to disaggregate proteins only at high concentrations, it seemed (*73*) that the active constituent of urea might be the cyanate (**11**) that it forms in aqueous solution. Sodium cyanate was then shown (*73, 74*) to carbamoylate hemoglobin extensively, largely at the $NH_2$ terminal amines of the α and β chains and to be an inhibitor of sickling. Subsequently

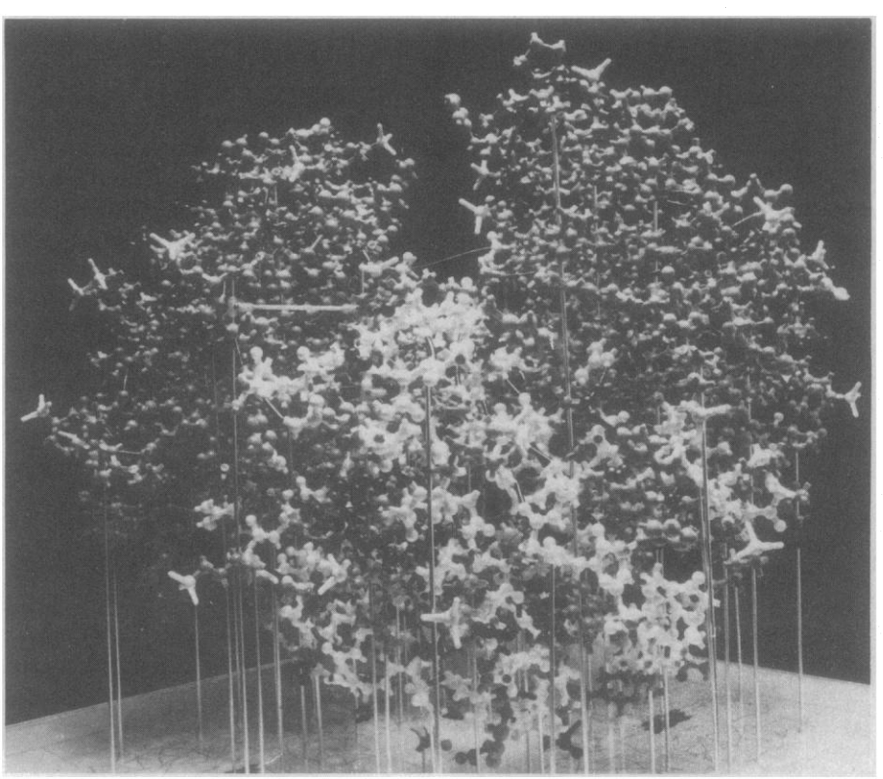


Fig. 4. Side view of scale molecular model of human deoxyhemoglobin showing β cleft, top center. [Model assembled by John Mack, photograph taken by Kevin Fishback of Sieber and McIntyre, Inc.]

Table 1. Percentage of hemoglobin modification and cross-linking by diaspirins.

| Compound | Percent modification* | | | β chains cross-linked (%) |
|---|---|---|---|---|
| | Intracellular | | Extracellular: one dose | |
| | One dose | Four doses | | |
| *Bis(salicyl) diesters* | | | | |
| 1 Fumarate (unsaturated $C_4$) | 0 | 0 | 70 | 85 |
| 2 Succinate ($C_4$) | 0 | 0 | 14 | 10 |
| 3 Adipate ($C_6$) | <5 | 15 | 16 | 0 |
| 4 Suberate ($C_8$) | 10 | 35 | 14 | 0 |
| 5 Sebacate ($C_{10}$) | 24 | 42 | 14 | 0 |
| 6 Dodecanedioate ($C_{12}$) | 35 | 65 | 17 | 0 |
| *Bis(3,5-dibromosalicyl) diesters* | | | | |
| 7 Fumarate (unsaturated $C_4$) (1 m*M*) | 15 | 93 | 88 | 70 |
| 8 Succinate ($C_4$) (1 m*M*) | 18 | 64 | 70 | 40 |
| 9 Sebacate ($C_{10}$) (0.5 m*M*) | 29 | 63 | 30 | 0 |

*Hemoglobin in oxygenated form.

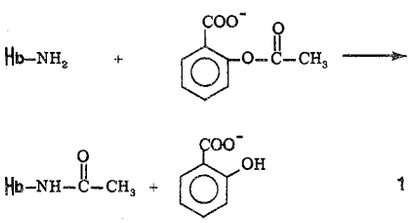
a double-blind clinical study revealed no improvement in sickle cell crises and gave evidence of toxicity (*75, 76*).

Turning to acylating agents, one can readily suggest many anhydrides or acid chlorides. But these have no likelihood of being pharmacologically acceptable.

2) *Aspirins*. The particularly novel idea that occurred to us a few years ago (*38*) was to use acylsalicylates, of which aspirin (**21**) is the prototype, as especially mild acylating reagents (*77*) for attaching various groups to the Lys and primary amino groups of hemoglobin. Aspirin is known to react with small molecule amines and had been reported (*78*) to acetylate serum albumin in vivo. With $^{14}C$-labeled aspirin we were able to demonstrate (*38*) acetylation of hemoglobin within erythrocytes, as well as in hemolyzates. That aspirin acetylates hemoglobin was confirmed by others (*79, 80*). Such acetylation also occurs in vivo, as is evident from the observations of Bridges *et al.* (*80*) with aspirin-treated patients who have been exposed to multiple doses. However, the effect of a single exposure to aspirin on sickling of HbS erythrocytes (in vitro) is very small. It is apparent that acetylsalicylate in itself is not acylating hemoglobin S to a sufficient extent to produce a marked physiological effect.

Aspirin, however, is only the simplest of the broad class of acylsalicylates. Increases in acylating activity, and hence of antisickling effect, might be achieved by appropriate modification of either the leaving group (salicylate) or the acyl function.

*Monosalicylates*. Aspirin acetylates amino groups of intracellular or extracellular hemoglobin by an aminolysis reaction (Eq. 1).




(1)

To increase the reactivity of alternative aspirins, we need to increase the electrophilicity at the ester bond. The salicylate moiety is generally the major determinant of the reactivity of acylsalicylates. One way to increase the lability of these esters is to place electron-withdrawing substituents on the aromatic phenolic ring. This can readily be achieved with a series of mono- and dihalogenated derivatives (**23** to **30**). Since dibromosalicylic acid is readily available commercially, we synthesized first *O*-acetyl-3,5-dibromosalicylate (**29**), "dibromoaspirin." The reaction of 5 m*M* dibromoaspirin with a suspension of normal erythrocytes (*p*H 7.2 at 37°C for 2 hours) led to the modification of 70 to 80 percent of the hemoglobin. At these low concentrations, ordinary aspirin yields no detectable modification.

Modification of sickle erythrocytes with dibromoaspirin produced a substantial decrease in erythrocyte sickling. A decrease in the number of abnormally shaped cells other than the characteristic sickled form was also apparent.

If the increased effectiveness of dibro-

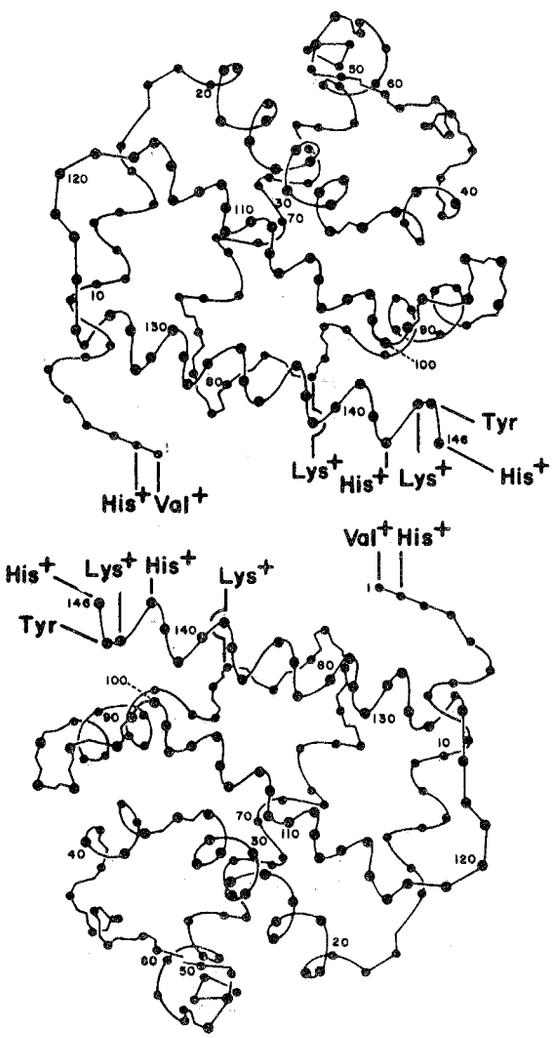


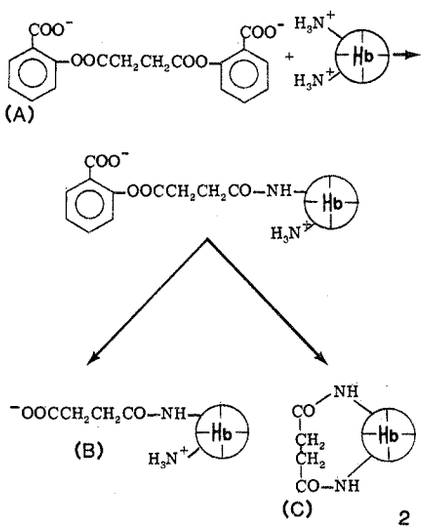

Fig. 5. Projection of three-dimensional arrangement of both β chains of hemoglobin. [Adapted from Muirhead and Perutz (*33*) and Arnone (*83*)]

moaspirin, as compared to aspirin, is due to the electron-withdrawing effect of the bromine atoms, then a chlorine or fluorine derivative should be even more potent. On the other hand, the augmentation in effectiveness may be a manifestation of increased binding of the dibromoaspirin to hemoglobin because of increased polarizibility interactions. In that case, the chlorine or fluorine derivatives of aspirin should be less efficacious, an iodine analog more so. For this reason we set up the two series of derivatives, **22** to **26** and **27** to **30**.

The extent of modification of hemoglobin by the monohaloaspirins (*81*) decreased in the order I > Br > Cl > F. Evidently increased binding must be the dominant factor in reactivity; fluoroaspirin should have been the most potent if electron-withdrawing ability were the crucial feature. With dihaloaspirins the distinction between derivatives is negligible; apparently with two halogen substituents, binding of the aspirin approaches saturation and differences in activity cannot be discerned.

*Double-headed aspirins: bis-salicylates*. A second and different covalent strategy can also be envisaged in which the conformation of HbS molecules is perturbed so that potentially interacting groups at the contact interfaces are pulled out of register and their interactions weakened indirectly. For this approach we recognize first that a central cavity exists along the dyad axis of the quaternary structure of hemoglobin (Fig. 4). This cavity has a shape somewhat like that of two adjoining boxes, each about 20 Å long perpendicular to the dyad axis, 8 to 10 Å wide and 25 Å deep along the dyad axis (*33, 34*). One box separates the α chains, the other the β chains. It is in the portal between the β chains (Fig. 4) that organic phosphate ions, such as 2,3-diphosphoglycerate, fit and modulate oxygen affinity (*82, 83*). This internal cavity is lined with cationic polar residues, the α-$NH_3^+$ of β1 Val, the imidazoles of β2 His and of β143 His and the ε-$NH_3^+$ of β82 Lys (*83–85*). Eight such residues are supplied by the two β chains. Of these, four provide $-NH_2$ groups, two on each β subunit, that can be acylated. If anionic, double-headed aspirins could be designed, they would have a high probability of being bound (noncovalently) within the cationic β cleft and of reacting (covalently) with suitably spaced $-NH_2$ groups on opposite β chains. Our second general program for developing alternative acylsalicylates has focused on diaspirins of this type.

That this concept works was first illustrated in exploratory experiments (*86*) with bis(*o*-carboxyphenyl) succinate (**31**, $n = 2$), two salicyl groups joined by $COCH_2CH_2CO$. Such bis(salicyl) esters of dicarboxylic acids could react with hemoglobin as follows:




The intermediate A would not persist since it is susceptible to hydrolysis, forming B, or to aminolysis by a second amine appropriately positioned to form a cross-linked species C. If a cross-link arises between amino groups on different subunits of the hemoglobin tetramer, stable dimers will persist under conditions that normally dissociate native hemoglobin into constituent α and β monomers. The extent of such intersubunit cross-linking, therefore, can be assessed by techniques that distinguish proteins on the basis of size, such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

In the bis(salicyl) diester series (**31**), we (*87*) have synthesized compounds with $n = 2, 4, 6, 8$, and 10 to give di-acid bridges with spans of $(n + 2)$ carbon atoms. In addition, a bis(salicyl) fumarate (**32**) was prepared to give a bridge with a rigid conformation. In view of the increased reactivity of dibromosalicylate, as compared to salicylate, we have also synthesized bis(3,5-dibromosalicyl) diesters of succinic (**33**, $n = 2$), sebacic (**33**, $n = 8$), and fumaric (**34**) acid, respectively.

Among bis(salicyl) diesters (compounds 1 to 6 of Table 1), the fumarate derivative (compound 1) produced vastly superior acylation of extracellular hemoglobin. Its effectiveness probably arises from the facilitation of electron withdrawal from the reacting acyl C=O and from resonance stabilization of the anion intermediate of aminolysis provided by the conjugated double bond. Such assistance is not furnished by the diesters derived from saturated dicarboxylic acids (compounds 2 to 6), all of which exhibited lower, roughly equivalent levels of modification extracellularly. The differences in intracellular acylation reflect increases in permeability with the longer chain compounds (*88*).

The bis(*dibromo*salicyl) diesters (compounds 7 to 9 in Table 1) were generally much more reactive than the corresponding nonbrominated diesters (compounds 1 to 5 in Table 1), a finding that parallels that found for the monoester derivatives. Furthermore, the presence of the bromine substituents increased the membrane permeability of these reagents so that the four-carbon brominated derivatives (compounds 7 and 8) provided significant acylation of intracellular hemoglobin in marked contrast to the corresponding nonbrominated compounds. As with the nonbrominated diesters, the fumarate diester (compound 7) proved most reactive in the series of brominated compounds. Also intracellular modification rose with increase in chain length of the acyl group to $C_{10}$.

Inasmuch as the diesters (compounds 1 to 9) contain two activated acyl functionalities, each susceptible to nucleophilic attack by amines, these derivatives have the potential to cross-link sites of hemoglobin. Table 1 shows the extent of monomer cross-linking for each diester (*87*). With nonbrominated diesters, only four-carbon acyl groups (compounds 1 and 2) provided cross-linking bridges. Among the brominated esters, the fumarate (compound 7) and succinate (compound 8) were very effective.

In all cases examined, cross-linking by four-carbon diesters occurred exclusively between β subunits within the hemoglobin tetramer. The bridges formed thus must be in some area where the two β subunits are in close approximation. This is the region of the β cleft.

A side view of the β cleft on a scale molecular model of hemoglobin is shown in Fig. 4. It is obvious that this molecular canyon is very deep. Furthermore, its walls are lined with cationic residues (Fig. 5). One would expect such a region to have a strong affinity for organic anions (*89*) such as the bis-salicylates. Within the β cleft of deoxyhemoglobin the distances between pairs of amino groups on opposite β chains (Fig. 5) are as follows: $\beta_1 82$ Lys $\cdots$ $\beta_2 82$ Lys, 8.1 Å; $\beta_1 82$ Lys $\cdots$ $\beta_2 1$ Val, 11 Å; $\beta_1 1$ Val $\cdots$ $\beta_2 1$ Val, 18 Å. Molecular models supply distances between nitrogen atoms attached to opposite ends of the fully

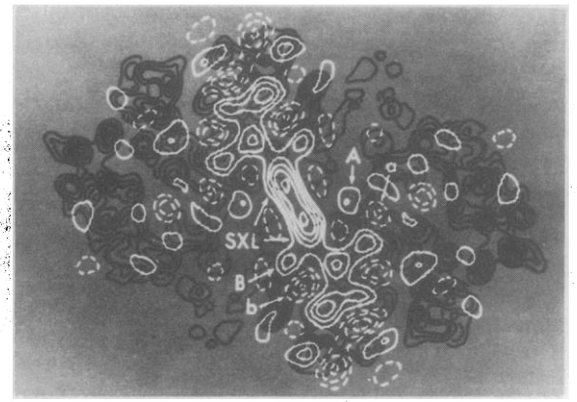



Fig. 6. Electron density map of deoxyhemoglobin looking down into the β cleft (*90, 91*). Difference electron densities of succinate–cross-linked versus native hemoglobin shown as white contours. [Courtesy of *Journal of Molecular Biology*]

extended dicarboxylic acids examined in our studies: -CO-CH=CH-CO-, 6.8 Å; -CO-$(CH_2\text{-}CH_2)_n$-CO-, 6.8, 9.2, 11.6, 14.0, and 16.4 Å for $n$ = 1, 2, 3, 4, and 5, respectively. The 1 Val · · · 1 Val and 82 Lys · · · 1 Val distances are large for the span of the $C_4$ diesters, and consequently an 82 Lys → 82 Lys bridge should be favored.

That this is indeed the site of cross-linking has now been established experimentally (*90, 91*). X-ray diffraction has shown that the fumaryl or succinyl group spans $\beta_1$82 Lys to $\beta_2$82 Lys (Fig. 6). The difference electron density map (white contours in Fig. 6) shows the strong electron density of the succinyl group spanning the β cleft, as well as areas in the EF helical bend that are pulled out of register compared to unmodified hemoglobin. Furthermore, this cross-link increases the solubility of the modified deoxy HbS and thus inhibits its aggregation directly (*91*).

As this survey illustrates there are many rational molecular strategems for modifying sickle hemoglobin so as to reduce its sickling tendencies. A chemist in his hubris might think that once a compound has been found that decreases aggregation of hemoglobin the problem, in principle, has been solved. However, we must recognize at least two other crucial features: (i) ability to penetrate the membrane, (ii) selectivity for hemoglobin over other proteins.

Our understanding of the molecular bases of membrane permeability, passive or energetically active, is still at a very rudimentary stage, in large part because so little is known about details of molecular constitution and structures of any membrane. With the aspirins that we have examined, we were hopeful and pleasantly surprised that the brominated diaspirins, in contrast to the nonbrominated ones, would penetrate the membrane but we would not have predicted such behavior with any assurance. But once the facts are known we can rationalize them in terms of molecular pictures or mechanisms.

Furthermore, if a compound penetrates a red cell membrane it may also penetrate the membranes of many other cells. Then we are faced with the problem that the substance may interact with other proteins, or biological metabolites, quite contrary to our intentions. For example, aspirin penetrates platelet membranes and acetylates enzymes within. It also reacts with albumin in the blood serum without having to penetrate a membrane. Fortunately, experience of about a century indicates that the hazards of these side reactions are not severe enough to overbalance the advantages of the analgesic effects. To some extent the probability of undesirable side effects can be minimized by designing the drug to fit specifically within the receptor site of the target protein. Thus the $C_4$ diaspirins have functional groups sterically disposed to be accommodated well within the β cleft of hemoglobin. We hope, therefore, that no other human protein has a site whose structure complements that of this reagent and that no other protein will bind diaspirins. If we had complete molecular knowledge of all the proteins in the human organism, we might, in principle, be able to design our drug to behave this way. However, absolute specificity is probably nonexistent. For example, the β cleft of hemoglobin binds molecules with a wide range of structures, and free energies, even though only one substance, 2,3-diphosphoglycerate, is presumed to be the physiologically significant one. Binding is not an all-or-none phenomenon. Even with a poor steric fit between effector and receptor site, if there are any interactions, there will be a free energy change accompanying binding. A standard free energy change of zero still corresponds to an equilibrium constant of 1. We will always be faced with the dilemma that in a complex, highly coupled homeostatic system, the introduction of a perturbing reagent is likely to create risks as well as benefits. Nevertheless, with a rational program focusing on molecular specificity, we can hope to minimize the former and maximize the latter.

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# Producer Gas Engines in Villages of Less-Developed Countries

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*Summary.* Producer gas engines could have an important role in the decentralized production of mechanical energy in rural areas of less-developed countries. With this technology mechanical energy is produced from solid fuels by use of internal combustion engines. A comparison with other renewable energy options, on the common basis of energy efficiency and economics, shows that producer gas engines may have significant advantages and deserve serious attention.

Access to mechanical power is needed in the villages of less-developed countries (LDC's) for lift irrigation, ploughing, threshing, transportation, and other uses. Traditionally, people in LDC's have obtained a major share of their mechanical energy from draft animals, at a very low overall thermal efficiency of 3 to 5 percent (*1*). A comparison of the energy efficiency of agricultural production showed that LDC's use more energy per unit of production than do developed countries. For example, in India 20 gigajoules are needed to produce 1 metric ton of rice, compared to 6.5 GJ to produce the same crop in Japan and the United States (*1*). However, Japanese and U.S. farmers use high-quality, nonrenewable energy sources such as petroleum and natural gas, whereas virtually all the energy needed by Indian farmers is derived from crude agricultural residues and other biomass.

Until recently, some farmers could increase their energy efficiency through the use of petroleum-based machinery. As petroleum becomes more expensive and less readily available, this option becomes less attractive. Fossil fuel–poor LDC's must therefore seek to increase the energy efficiency of using renewable energy resources such as wood, crop residues, dung, and solar radiation. Given their constraints of capital, the diffuse nature of the resources, and lack of technology and know-how, this is not an easy task. For example, photovoltaic power generation is not economically feasible with current technology. Wind power and hydropower are site-specific and generally limited to stationary applications. Production of methanol and ethanol from biomass is still energy-inefficient and capital-intensive. Small steam engines have low efficiency (<10 percent). Stirling engines need a major development effort to make them cost-effective.

By contrast, the producer gas engine, which is an internal combustion (IC) engine, has several advantages. It can run on solid fuels such as wood, straw, and other crop residues; it has a moderately high engine efficiency (20 to 30 percent), a low cost, and is easily adaptable to existing IC engines. Moreover,

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