Structural Features Required for the Reactivity and Intracellular Transport of Bis(3,5-dibromosalicyl)fumarate and Related Anti-sickling Compounds That Modify Hemoglobin S at the 2,3-Diphosphoglycerate Binding Site*

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Bis(3,5-dibromosalicyl)fumarate (I) reacts preferentially with oxyhemoglobin to cross-link the two 882 lysine residues within the 2,3-diphosphoglycerate (DPG) binding site and as a result markedly increases the solubility of deoxyhemoglobin S. The cross-link acts by perturbing the acceptor site for Val 8 within the sickle cell fiber (Chatterjee, R., Walder, R. Y., Arnone, A., and Walder, J. A. (1982) Biochemistry 21, 5901–5909). In the present studies we have compared a large number of analogs of I to determine the structural features of the reagent required for specificity and for transport into the red cell. Both electrostatic and hydrophobic interactions contribute to the binding of these compounds at the DPG site. The optimal position for the negatively charged groups on the cross-linking agent for productive binding is adjacent to the ester as in the original salicylic acid derivatives. There is a direct correlation between the reactivity toward hemoglobin and the hydrophobicity of the substituent attached at the para position. Phenyl and substituted phenyl derivatives as in the analgesic, anti-inflammatory drug diflunisal are particularly effective. These groups probably interact with hydrophobic residues of the amino-terminal tripeptide and the EF corner of the β chains adjacent to the DPG binding site.

Although bis(3,5-dibromosalicyl)fumarate is very reactive toward hemoglobin in solution, it is much less effective in modifying hemoglobin within the red cell. The reaction with intracellular hemoglobin was shown to be limited by competing hydrolysis of the reagent catalyzed at the outer surface of the erythrocyte membrane. Inactivation of the red cell membrane acetylcholinesterase with phenylmethylsulfonyl fluoride did not inhibit this reaction. Introduction of a single methyl group onto the carbon-carbon double bond of the fumarate moiety decreases the lability of the ester 10-fold, due to steric effects, and allows the reagent to be taken up by the red cell and modify intracellular hemoglobin. The kinetics of transport of the methyl-fumarate derivative, bis(3,5-dibromosalicyl)mesaconate, are first-order, consistent with passive diffusion. The attachment of larger alkyl groups onto the cross-link bridge further enhances the transport of the reagent into the red cell. The solubility of deoxyhemoglobin S cross-linked with the butylfumarate derivative was found to be increased by almost 10% compared to the original fumarate diester. The added substituent attached to the bridging group may displace the amino-terminal tripeptide and A helix of the β chains away from the central cavity of the hemoglobin tetramer and directly perturb the mutation site at Val 6. Pharmacokinetic studies indicate that high levels of modification of intracellular hemoglobin may be achieved with the substituted fumarate derivatives at steady-state concentrations of the drug between 100 and 200 μM. Red cells modified in vitro under these conditions with bis(3,5-dibromosalicyl)mesaconate have nearly normal viability.

In concentrated solutions, as within the erythrocyte, deoxyhemoglobin S polymerizes to form extended helical fibers. These fibers align parallel with each other to form large aggregates which distort the red cell membrane, giving rise to the characteristic sickled shape. The intracellular hemoglobin polymer markedly reduces the deformability of the erythrocyte. As a result, such cells tend to become trapped within the small blood vessels of the microcirculation causing the vaso-occlusive complications of sickle cell disease.

The deoxyhemoglobin S fiber is composed of a series of double-stranded filaments, alternating in polarity, in which the two strands of hemoglobin tetramers are related by an approximate 2-fold screw axis (1–5). This double-stranded structure was first observed by Love and his co-workers in single crystals of deoxyhemoglobin S (1). The analysis of the crystal structure has provided a detailed image of the most important set of intermolecular contacts which stabilize the fiber. The mutation site, Val 6β, is involved in the lateral contact between the two hemoglobin strands. The side chain of the valine residue extends into a hydrophobic pocket formed by the Phe 85β and Leu 88β of an adjacent tetramer. This thermodynamically favorable interaction is the principal basis for the decrease in the solubility of deoxyhemoglobin S and has become the main target for the design of stereospecific reagents to interfere with the aggregation process.

From initial studies of a large number of acyl salicylates as potential anti-sickling compounds (6–10), work in our laboratory has focused on a novel class of affinity reagents that react with hemoglobin at the DPG¹ binding site and cross

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¹ The abbreviations used are: DPG, 2,3-diphosphoglycerate; DIDS, 4,4'-diisothiocyanato-2,2'-disulfonic acid; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
link the β chains between the two β82 lysine residues (11, 12). The original compounds in this series, bis(3,5-dibromosalicyl)fumarate and bis(3,5-dibromosalicyl)succinate, introduce a four-carbon cross-link at this site (Equation 1). As a result of this modification, the solubility of deoxyhemoglobin S is increased by nearly 50% (11, 12), an effect much larger than that thought needed to be clinically beneficial based on comparison with other sickle hemoglobinopathies (13).

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{Br} \\
\text{Br} & \quad \text{NH}_2 \\
\text{C} & \quad \text{C} \\
\text{O} & \quad \text{O} \\
\text{C} & \quad \text{C} \\
\text{O} & \quad \text{O} \\
\text{Br} & \quad \text{Br} \\
\text{H} & \quad \text{H} \\
\text{Br} & \quad \text{Br} \\
\text{Br} & \quad \text{Br} \\
\end{align*}
\]

**EQUATION 1**

The reaction of bis(3,5-dibromosalicyl)fumarate with lysine 82β occurs specifically with oxyhemoglobin. In the liganded quaternary state the disposition of the two lysine residues is correct for cross-linking to occur without perturbing the protein structure (12). This complementary stereochemistry is an important determinant of the specificity of the reagent. In the transition to deoxyhemoglobin the β chains move apart and the distance between the α carbon atoms of the two β82 lysine residues is increased by more than 5 Å (14–16). With the cross-link in place, the extent of this movement is limited. X-ray studies of the cross-linked derivative showed that Lys 82 and a large portion of the F helix (β chain residues 85–95) are displaced inward toward the central cavity of the tetramer relative to their positions in the native deoxy structure (11, 12). Phe 85β and Leu 88β, the residues which form the acceptor site for Val 6 within the deoxyhemoglobin S fiber, are located at the beginning of the F helix. The displacement of these residues away from the surface of the molecule weakens the interaction with Val 6 within the fiber and accounts for the increase in the solubility of deoxyhemoglobin S. There is no perturbation of the structure discernible in the region of the mutation site. As the length of the bridging group is increased from four to six carbon units, the limits over which cross-linking occurs, the constraint on the structure is relaxed, and the magnitude of the displacement of Lys 82 and the F helix becomes progressively less (12). Correspondingly, the solubility decreases, approaching that of native deoxyhemoglobin S, in agreement with the proposed stereochemical mechanism (12).

Even in the case of the fumarate and succinate derivatives, the constraint imposed by the cross-link leads only to local perturbations in the structure. Overall the quaternary structure is essentially identical to that of native deoxyhemoglobin. Similarly there is relatively little change in the p50 or decrease in the cooperativity of oxygen binding (11, 17). The cross-link does block the DPG binding site, and as a result increases the oxygen affinity of intracellular hemoglobin. This may be beneficial in sickle cell disease, and earlier has been proposed as a means of therapy (18, 19). Alternatively this effect may be mitigated by attaching a negatively charged group onto the cross-link bridge, acting as a permanently bound anion, as in the case, for example, of 2-nor-2-formyl pyridoxal phosphate (20).

Here we report the synthesis and characterization of a number of new analogs of bis(3,5-dibromosalicyl)fumarate and -succinate to determine the structural features of the reagent required for optimal specificity toward hemoglobin and transport into the red cell.

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**EXPERIMENTAL PROCEDURES**

**Organic Synthesis**

*Materials—* 5-(2',4'-Difluorophenyl)salicylic acid (difuinosal), was obtained from Merck Sharp and Dohme. All other reagents used as starting materials in the syntheses were purchased from Aldrich. Bis(saliclyl)succinate (1), bis(3,5-dibromosalicyl)succinate (16), and bis(3,5-dibromosalicyl)fumarate (17) were available from earlier studies in this laboratory (11, 12).

**General Procedures***—Melting points were determined with a Thomas capillary melting point apparatus and are uncorrected. Proton nuclear magnetic resonance spectra were obtained with a Varian 360L (60 MHz) spectrometer. Chemical shifts are reported in parts/million (δ) downfield from tetramethylsilane. Elemental analyses were done by Galbraith Laboratories, Inc., Knoxville, TN.

*Bis(m-carboxyphenyl)succinate* (2)—m-Hydroxybenzoic acid (4.14 g, 30 mmol) was dissolved in 25 ml of water with 2 equivalents of NaOH and chilled in an ice bath to 0 °C. Succinyl chloride (16.5 mmol) was then added dropwise to the solution with vigorous stirring over 3 min. The reaction was allowed to proceed at 0 °C for 15 min. The mixture was then diluted with 2 volumes of cold water and acidified with concentrated HCl (35 mmol) to give a white precipitate. The solid was filtered, washed with water, and then air-dried. Recrystallization from methanol/water gave the pure product in 65% overall yield (3.91 g), m.p. 236–237 °C. C_{19}H_{14}O_5

Calculated: C 60.32 H 4.10

Found: C 60.41 H 4.09

**Compounds 3, and 5–7 were prepared in the same manner.**

*Bis-(p-carboxyphenyl)succinate* (3)—Recrystallization from methanol gave the pure compound in 68% yield, m.p. 265 °C (decomposed).

C_{19}H_{14}O_5

Calculated: C 60.32 H 4.10

Found: C 60.32 H 4.10

*Bis(m-carboxymethylphenyl)succinate* (5)—The product was isolated in 71% yield after recrystallization from methanol/water, m.p. 163–164 °C.

C_{23}H_{20}O_5

Calculated: C 62.18 H 4.70

Found: C 62.12 H 4.71

*Bis(p-carboxymethylphenyl)succinate* (6)—The product was obtained in 78% yield after recrystallization from methanol, m.p. 211–214 °C.

C_{23}H_{20}O_5

Calculated: C 62.18 H 4.70

Found: C 62.21 H 4.76

*Bis(o-trans-cinnamyl)succinate* (7)—After isolation of the product, the solid was extracted with boiling ethanol (56%), m.p. 231–232 °C.

C_{23}H_{20}O_5

Calculated: C 64.39 H 4.42

Found: C 64.21 H 4.62

**Benzyl-o-hydroxyphenylacetate—** A mixture of o-hydroxyphenyl acetic acid (3.04 g, 20 mmol), benzyl alcohol (2.16 g, 20 mmol), and a catalytic amount of p-toluenesulfonic acid (∼50 mg) in toluene (100 ml) was heated to reflux for 24 h with a Barrett-type receiver. After cooling down to room temperature the solution was evaporated in vacuo to dryness. The residue was recrystallized twice from cyclohexane to give 4.50 g (93%) of the product, m.p. 101–102 °C, lit. 98–100 °C (21).

**Bis-o-carboxymethylphenylacetate** (4)—A mixture of benzyl o-hydroxyphenylacetate (2.42 g, 10 mmol), succinyl chloride (930 mg, 6.0 mmol), and pyridine (949 mg, 12.0 mmol) dissolved in toluene (100 ml) was refluxed for 2 h. After cooling down to room temperature, the mixture was evaporated in vacuo to a heavy syrup which was added to an ice-water mixture and extracted with chloroform. The organic layer was dried (Na_2SO_4) and then passed through a short Al_2O_3 column equilibrated with chloroform. Fractions containing the product were pooled and evaporated in vacuo to give a heavy syrup,
Design of Anti-sickling Compounds

790 mg (23%). The product was hydrogenated without further purification. The entire sample was dissolved in 20 ml of ethanol and glacial acetic acid (1:1) and hydrogenated at 45 p.s.i. in a Parr hydrogenator with a catalytic amount of 5% palladium on charcoal. After removing the catalyst, the solution was evaporated in vacuo and recrystallized twice from ethyl acetate to give 40 mg of the product, 3.0% overall yield, m.p. 141-142 °C; NMR (dimethyl sulfoxide-d$_6$) δ 3.0 (singlet, 4H, -CH$_2$CH$_2$), 3.53 (singlet, 4H, -CH$_2$-), 7.32 (multiplet, 8H, aromatic).

5-Alkyl- and Aryl-substituted Salicylic Acids—5-Ethylsalicylic acid, 5-propylsalicylic acid, and 5-phenylsalicylic acid were synthesized by carboxylation of the Na$^+$ salts of the corresponding phenols in refluxed chloroform (43%), m.p. 193-196 °C.

Bis(o-carboxyphenyl)succinate (8)—Bis(o-hydroxycinnamoyl)succinate (7) (1.51 g, 3.68 mmol) was suspended in a mixture of 100 ml of ethanol and 50 ml of tetrahydrofuran. Hydrogenation was carried out at 55 p.s.i. in a Parr hydrogenator using 300 mg of palladium on charcoal. During the course of the reaction the starting material dissolved. After 30 min the uptake of hydrogen ceased and the catalyst was filtered. The solution was evaporated to starting material dissolved. After 30 min the uptake of hydrogen ceased and the catalyst was filtered. The solution was evaporated to

Bis(5-fluorosalicyl)succinate (9)—succinyl chloride (10 mmol, 1.55 g) was added dropwise with stirring to a solution of 5-fluorosalicylic acid (20 mmol, 3.12 g) and N,N-dimethylaniline (40 mmol, 4.84 g) in dry benzene (50 ml) at room temperature. The resulting solution was stirred at room temperature in a stopped vessel overnight. Benzene was evaporated under reduced pressure and the residue was redissolved in acetone. The acetone solution was poured with vigorous stirring into an ice-water mixture containing a slight excess of HCl (~45-50 mmol). The mixture was stirred for 20 min. The solid product was filtered, washed with cold water, dried, and recrystallized twice from ethanol and chloroform to give the desired compound (2.14 g, 54%), m.p. 180-190 °C.

Bis(5-methylsalicyl)succinate (13)—Recrystallized from methanol (69%), m.p. 199-201 °C.

Bi5(5-iodosalicyl)succinate (12)—Recrystallized from ethanol/tetrahydrofuran (58%), m.p. 188-190 °C.

Bis(5-ethylysalicyl)succinate (14)—Recrystallized from methanol (51%), m.p. 203-204 °C.

Bis(5-propylsalicyl)succinate (15)—Recrystallized from ethyl acetate/hexane (59%), m.p. 172-175 °C.

Bis(5-ethylsalicyl)mesaconate (19)—Recrystallized from ethyl acetate/methanol (62%), m.p. 222-223 °C.

Bis(5-methylsalicyl)fumarate (20)—Recrystallized from ethyl acetate/methanol (45%), m.p. 217-218 °C.

Bis(5-iodosalicyl)2-n-butylfumurate (22)—Recrystallized from ethyl acetate/cyclohexane (38%), m.p. 224-225 °C.

Bis(5-bromosalicyl)fumarate

Bis(5-bromosalicyl)-2-n-butylfumarate

Bis(3,5-dibromosalicyl)d,L-2-methylysuccinate (18)—Recrystallized from ethanol/water (26%), m.p. 189-190 °C.

Bis(5,5-dibromosalicyl)-D,L-2-methylysuccinate (17)—Recrystallized from 1N HCl (57.5 mmol, 4.0 g) was dissolved in 800 ml of 50 mM Na$_2$CO$_3$ buffer, pH 10.0, and allowed to react for 3 h at room temperature. The solution was brought to 0 °C and acidified by the addition of 110 ml of 1 M HCl. A white oily precipitate formed which was first recrystallized from ethanol from which was recovered 0.45 g (11%) of the starting material and then recrystallized twice from ethanol/water (26%).
ethanol/chloroform to give the desired product (1.06 g, 48%); m.p. 224 °C (decomposed); NMR (dimethyl sulfoxide- $d_2$): $\delta$ 7.02 (apparent singlet, 2H, alkene) 8.17 (doublet, 1H, aromatic), 8.37 (doublet, 1H, aromatic).

C$_{10}$H$_{14}$Br$_4$O$_6$

Calculated: C 33.53 H 1.54 Br 40.56
Found: C 33.22 H 1.56 Br 40.79

**Chemical Modification Studies**

Hemoglobin A was isolated from fresh whole blood as described by Perutz (25) and stored as a 15% solution frozen in liquid nitrogen. Hemoglobin S was kindly provided to us by Dr. Robert Josephs and stored at -20 °C in 50% glycerol. Reactions of the cross-linking agent with oxyhemoglobin were carried out in 0.2 M bis-Tris chloride, pH 7.2, at 37 °C. In general, reactions with whole cells were performed with a 20% erythrocyte suspension in the same buffer. For more prolonged incubations in which the erythrocytes were repeatedly resuspended with a fresh solution of the compound, the buffer was 20 mM sodium phosphate, pH 7.2, 0.125 M NaCl and 10 mM glucose. The extent of cross-linking was determined by sodium dodecyl sulfate-gel electrophoresis (9). Isoelectric focusing and two-dimensional gel electrophoresis of the modified hemoglobins were carried out as described earlier (9, 12). To determine if the cross-linking agents enter the red cell through the anion transport channel, band 3, uptake through this pathway was blocked using the inhibitor DIDS (26). In these experiments red cells were first modified with 20 μM DIDS for 30 min at 37 °C in 0.5 M bis-Tris buffer, pH 7.2. After the incubation, the sample was centrifuged and the red cells were washed twice in normal saline. The reaction with Aldred (3,5-dibromosalicyl)fumarate and -succinate was then compared with untreated cells. To prove that the reaction with DIDS had blocked the anion channel, 10 μl of the treated cells were resuspended in a 1 ml solution containing 150 mM ammonium chloride, 50 μg of carbonic anhydrase, and 10 mM HEPES buffer at pH 7.4 (27). Cells treated with DIDS were stable for more than 1 h, whereas unmodified cells were completely lysed within 5 min. This assay was also used as a measure of anion transport activity in cells modified with the cross-linking agents alone.

**Transport Studies**

The rate of transport into the red cell and the kinetics of hydrolysis were followed directly for several of the compounds studied. In these experiments the reagent was adjusted to an initial concentration of 1 mM in a 20% suspension of red cells in 0.125 M NaCl and 20 mM sodium phosphate buffer, pH 7.2. All reactions were carried out at 37 °C. Aliquots were taken from the reaction mixture at various times and immediately centrifuged, and the concentration of the reagent and hydrolysis products in the supernatant was determined by high-pressure liquid chromatography with an analytical reverse phase CIS column (Altex). The columns were developed with a mobile phase of 70% 0.125 M NaCl and 20 mM sodium phosphate buffer at pH 7.2 and 30% methanol at a flow rate of 0.9 ml/min. Individual components were quantitated from graphical integration of the elution peaks recorded at 254 nm using a calibration curve determined with each of the pure compounds.

**Aminolysis with Proplyamine**

The reaction with propylamine (Equation 2) was used to compare the intrinsic reactivity of the esters toward amino groups. The course of the reaction was followed by measurement of the release of the phenol spectrophotometrically at the absorption maximum near 300 nm. The rates of aminolysis were determined at 37 °C in 0.02 M Tris chloride buffer, pH 8.5, with 150 mM NaCl, in the presence of a large excess of propylamine. Pseudo-first-order rate constants were determined from the initial rates. These values represent the sum of the microscopic rate constants for the reaction at each of the ester groups, which are obviously identical in the case of symmetric reagents. The second-order rate constant, $k_{obs}$, was determined from the slope of the line relating the observed pseudo first-order rate constants to the concentration of propylamine.

**Purification of Cross-linked Derivatives of Hemoglobin S and Solubility Studies**

For the isolation of hemoglobin S cross-linked with bis(3,5-dibromosalicyl)fumarate and -succinate, the protein was modified with a stoichiometric concentration of the reagent (1 mM) for 2 h at 37 °C in 0.2 M bis-Tris buffer at pH 7.2. The concentration of the compound was 2 mM for hemoglobin S cross-linked with bis(5-bromosalicyl)-2-n-butylfumarate. The cross-linked derivatives were first purified by chromatography on DEAE-cellulose as described previously (12). Minor remaining noncross-linked impurities were removed by gel filtration in the presence of 1 M MgCl$_2$ (12). Under these conditions derivatives that are not cross-linked dissociate to δδ dimers (29). The solubility of native deoxyhemoglobin S and of the cross-linked derivatives was determined at 28 °C in 0.15 M potassium phosphate buffer at pH 7.3 by ultracentrifugation using a Beckman air ultracentrifuge (12). The solubility of native deoxyhemoglobin S determined under these conditions was 19.8 ± 0.4 g/dl. The mean value and standard deviation were determined from 16 independent measurements. In order to reduce the error even further the cross-linked derivatives were centrifuged in the same rotor. The values reported in the text are the average of two separate experiments.

**Red Cell Survival Studies**

Male New Zealand White rabbits, approximately 2 kg in weight, were used for studies of the viability of red cells modified in vivo with bis(3,5-dibromosalicyl)mesaconate. 10 ml of blood was drawn from each rabbit via the ear vein into a sterile tube containing 10 μl of a 15% solution of EDTA. The blood was spun down in a clinical centrifuge, and 100 μCi of Na$^{51}$CrO$_4$ (New England Nuclear, specific activity 300-400 mCi/mg) was added to the plasma. The red cells were resuspended and the mixture was incubated at room temperature for 1 h. 100 μl of ascorbic acid (50 mg/ml), dissolved in sterile saline, was then added to reduce the chromium. After 30 min the sample was centrifuged and the cells were washed once in buffer (20 mM sodium phosphate, pH 7.2, 0.125 M NaCl, and 10 mM glucose) to remove unbound chromium. The red cells were resuspended in the same buffer and then treated with bis(3,5-dihydroxysalicyl)mesaconate under the various reaction conditions described in Table VII. Control cells were incubated under the same conditions in the absence of the compound. After the reaction the cells were washed several times and reinfused into the original donor as a 40% suspension in the reaction buffer. Approximately 10% of the sample was saved to correct for spontaneous radioactive decay. The first blood sample was drawn 1 day after the labeled cells were reintroduced. Samples were taken every 2-3 days thereafter, until the number of counts decreased to about 10% of the initial value (~10,000 cpm/ml of packed cells). At each time point 1 ml of blood was withdrawn and the radioactivity in 250 μl of packed cells was counted in a Beckman Biogamma counter. The 50% survival time was defined for these studies as the time required for the disappearance of 50% of the radioactivity, starting 1 day after injection of the labeled cells. Each of the values reported in Table VII is the average of 3 separate experiments.

**RESULTS AND DISCUSSION**

**Structural Features of the Leaving Group**—The series of analogs in Table I were compared to determine the optimal position of the negatively charged substituent on the leaving group. The original salicylic acid derivative, 1, in which the carboxyl group is ortho to the ester and attached directly to the aromatic ring, is clearly the most reactive toward hemoglobin. Aside from the para isomer, 3, there is no detectable cross-linking with any of the other reagents. The specificity for the ortho isomer is even greater when compared to the relative reactivity of the esters. For the model reaction with propylamine (Equation 2) the second-order rate constants for 1 and 3 are 4.9 X 10$^{-4}$ M$^{-1}$ min$^{-1}$ and 2.4 X 10$^{-4}$ M$^{-1}$ min$^{-1}$, respectively (the reaction conditions are described under “Experimental Procedures”). Due to steric and electrostatic effects of the carboxyl group substituted adjacent to the ester, the inherent reactivity of 1 is decreased about 5-fold compared to the p-carboxy derivative. A similar decrease in reactivity has been observed for the reaction of methyamine with the corresponding acetyl esters (29).
Design of Anti-sickling Compounds

Effect of the position of the carboxyl group on the reactivity of the cross-linking agent

| No. | Substituent | Position | Cross-linking |
|-----|-------------|----------|--------------|
| 1   | -COOH       | o        | 20           |
| 2   | -COOH       | m        | 0            |
| 3   | -COOH       | p        | 8            |
| 4   | -CH₃COOH    | o        | 0            |
| 5   | -CH₂COOH    | m        | 0            |
| 6   | -CH₂COOH    | p        | 0            |
| 7   | -CH₃CH₂COOH | o        | 0            |
| 8   | -CH₃CH₂COOH | o        | 0            |

*Modification reactions were carried out with 1 mM hemoglobin and 10 mM of the cross-linking agent at 37 °C for 4 h in 0.2 M bis-Tris buffer, pH 7.2. Per cent cross-linked product was quantitated from sodium dodecyl sulfate gels of the reaction mixture.

Although polyanions of widely different structures bind within the DPG site, for productive binding of the cross-linking agents the ester group must be in position to react with Lys 82. To determine the basis of the specificity for the salicylic acid derivatives, we have examined complexes of the carboxyl group in the salicylic moiety with either a sulfonate or phosphate group. However, due to the low pKₐ of these groups, the permeability of such compounds to the red cell membrane would probably be severely limited.

The addition of the halogen substituents to the aromatic ring in bis(3,5-dibromosalicyl)fumarate and -succinate, which is essential for the transport of these compounds into the red cell, also markedly increases their reactivity toward hemoglobin (9-11). In order to determine the basis for this effect, the series of para-substituted halogenated derivatives in Table II (compounds 9-12) were synthesized. The reactivity toward hemoglobin was found to increase monotonically with the size of the halogen substituent. This is the same order as observed for the hydrophobic properties of the halogens (31), and presumably relates to the increase in polarizability as the size of the halogen is increased. In contrast, there is very little difference in the intrinsic reactivity of the esters. For the model reaction with propylamine, the order of reactivity is Br > Cl > I > F, and there is only a 3-fold difference in the rate constants (see Table II). The contribution of hydrophobic binding is also seen in the series of para-substituted alkyl derivatives in Table II. In this case the inherent reactivity of the esters is decreased by about 2-fold due to the electron-donating effect of the alkyl group.

Table II

| No. | X         | Cross-linking | k₉ * |
|-----|-----------|---------------|------|
| 1   | H         | -             | 0.40 |
| 9   | F         | 6             | 0.56 |
| 10  | Cl        | 11            | 1.3  |
| 11  | Br        | 35            | 1.7  |
| 12  | I         | 60            | 1.1  |
| 13  | CH₃       | 7             | 0.24 |
| 14  | CH₃CH₂    | 12            | 0.23 |
| 15  | CH₃CH₂CH₃ | 20            | 0.23 |

*Reactions were carried out at 37 °C in 0.2 M bis-Tris buffer, pH 7.2, with the concentration of hemoglobin equal to 0.2 mM. In the first series the concentration of the compound was 2 mM and the reactions were carried out for 4 h. In the second series the concentration of the compound was 0.5 mM and the reactions were allowed to proceed for 10 h.

k₉ is the second order rate constant for the reaction with propylamine. Rate constants were determined at 25 °C in the presence of 0.02 M Tris buffer, pH 8.5, containing 150 mM NaCl.

*These values were too low to accurately quantitate.

Correlation of reactivity with hydrophobic substituents attached to the leaving group

Equation 2

With the above constraints, when the ester group is in position for attack by Lys 82, the carboxyl group in 1 is associated with a cluster of positively charged groups formed by Lys 144β, His 143β, and the amino-terminal valine residue of the β chain. Comparable interactions are not possible with the carboxyl group in the meta or para positions. Although similar interactions appear to be favorable for 4, in this case the ester is rapidly hydrolyzed probably due to intramolecular nucleophilic attack by the carboxyl group. Because of the polyanion nature of the electrostatic binding, it may be possible to further increase the affinity for the reagent by substitution of the carboxyl group in the salicylic moiety with either a sulfonate or phosphate group. However, due to the low pKₐ of these groups, the permeability of such compounds to the red cell membrane would probably be severely limited.
formed between residues of the amino-terminal tripeptide (Val 1 and Leu 3) and the EF corner (Leu 78 and Leu 81) of the β chains. The flexibility of the amino-terminal tripeptide, which in oxyhemoglobin is highly disordered (32, 33), should allow a variety of different apolar groups to be accommodated in this position. As shown below, phenyl derivatives attached to the salicylic acid group have been found to be particularly effective in promoting the reactivity of the cross-linking agents with hemoglobin (see Table VI).

There were no differences found in the reactivity of the compounds studied between hemoglobin A and hemoglobin S. The side chain of the valine residue at β6 in hemoglobin S extends into the solvent and is not in position to interact with apolar groups attached to the cross-linking agent.

**Comparison of the Specificity of Fumarate and Succinate Analogs**—The structures of deoxyhemoglobin A cross-linked by bis(3,5-dibromosalicyl)fumarate and -succinate are iso- morphous (11), and the solubilities of the corresponding derivatives of deoxyhemoglobin S are identical; both increased approximately 50% compared to native deoxyhemoglobin S (Table III). There is, however, a large difference in the reactivity of the two compounds. The rate constant for the reaction of bis(3,5-dibromosalicyl)fumarate with propylamine is increased by more than 80-fold compared to the succinate diester (see Table V). The greater reactivity of the fumarate diester is due to the electron-withdrawing effect of the opposing ester groups transmitted by the carbon-carbon double bond. Once one ester group is hydrolyzed, the second is much more resistant to nucleophilic attack (see below). In the succinate derivative, the methylene groups of the cross-link bridge effectively isolate the two ester groups.

Despite the much greater reactivity of the fumarate derivative, this compound also reacts more selectively with hemoglobin than the succinate derivative. The greater reactivity of the fumarate diketene in oxyhemoglobin and that cross-linking of the two p82 lysine residues occurs without perturbing the protein structure (12). Although the succinyl group can adopt essentially this same conformation upon binding within the DPG site, whatever internal rotational motion that existed in the free substrate becomes completely frozen out. This loss of rotational entropy makes an unfavorable contribution to the free energy of association relative to the fumurate diester, which is already fixed in conformation by the carbon-carbon double bond, and decreases the binding affinity. The importance of structural disposition of the bridging group has also been pointed out by Klotz and co-workers (34, 35), although the relative specificity of the various reagents studied was not determined.

With the idea of limiting the internal rotational motion of the succinyl group, we prepared a number of alkyl-substituted succinate derivatives. The substitution of even a methyl group onto the cross-link bridge substantially increases the torsional barrier to internal rotation about the central carbon-carbon bond. The predominant effect observed, however, was steric hindrance to nucleophilic attack of the ester, markedly reducing the reactivity toward hemoglobin. Under the same conditions as in Fig. 1 the yield of the cross-linked derivative with bis(3,5-dibromosalicyl)-2-methylsuccinate is only 11% compared to 45% for the original succinate diester. A similar decrease in reactivity is observed with model compounds. The rate constant for the reaction of bis(3,5-dibromosalicyl)-2-methylsuccinate with propylamine (2.5 \( \times \) \( 10^{-2} \) M\(^{-1}\) min\(^{-1}\)) is decreased nearly 10-fold compared to bis(3,5-dibromosalicyl)succinate (2.2 \( \times \) \( 10^{-1} \) M\(^{-1}\) min\(^{-1}\)). This large decrease in reactivity indicates that the steric effect of the alkyl substituent must extend to both ester groups. The rate constant determined from the initial velocity of the reaction represents the sum of the microscopic rate constants for the reaction of propylamine with each of the ester groups. Therefore, if the added methyl group affected the reactivity only at the adjacent carbon, the observed rate constant would be decreased by at most 2-fold. To account for a 10-fold decrease in the rate constant, the reactivity of the second ester group must be decreased by at least 5-fold. Examination of space-filling models indicates that steric hinderance is due predominantly to interactions with the substituent attached onto the incoming nucleophile. Since more complex modifications of the succinyl group would likely inhibit the reactivity even further, the fumaryl group appears to represent the optimal structure for the backbone of the cross-link bridge.

**The Mechanism of Transport across the Erythrocyte Membrane**—The correlation between the lipophilic properties of the halogen substituents and their ability to enhance the transport of the cross-linking agents across the red cell membrane first suggested that these compounds enter the red cell by passive diffusion (11). Similar correlations have been observed, however, for the transport of compounds by the anion channel of the erythrocyte membrane, band 3 (36). As shown in Table IV, pretreatment of erythrocytes with DIDS which irreversibly blocks the anion channel (26), did not affect the level of modification of intracellular hemoglobin with either bis(3,5-dibromosalicyl)fumarate or -succinate. This rules out the anion channel as the mechanism of transport. We have also shown that neither of these compounds inhibit band 3, using the rate of red cell lysis in the presence of NHCl as a measure of activity of the anion channel (27). This level of specificity of the reagent is important since an inhibition of anion transport in vivo would interfere, at least in part, with the export of CO₂.

Although bis(3,5-dibromosalicyl)fumarate is more reactive than the succinate analog toward hemoglobin in solution, it is much less effective in modifying hemoglobin within the erythrocyte (see Table IV). Since the level of modification of hemoglobin in crude hemolysates is the same with both of these compounds as with purified hemoglobin (data not shown), this difference cannot be due to a competing reaction with another constituent within the red cell. Rather, as shown

### Table III

**Solubility of cross-linked derivatives of deoxyhemoglobin S**

| Hemoglobin          | Solubility (g/dl) |
|---------------------|-------------------|
| Native hemoglobin S | 19.8              |
| Hemoglobin S (succinate) | 30.9            |
| Hemoglobin S (fumarate) | 31.1            |
| Hemoglobin S (n-butylfumarate) | 32.7            |
Design of Anti-sickling Compounds

FIG. 1. Two-dimensional gel electrophoresis of the cross-linked hemoglobins. A, native hemoglobin A. B, hemoglobin A modified with bis(3,5-dibromosalicyl)succinate. The Lys 82 → Lys 82 β dimer is indicated by the arrow. C, hemoglobin A modified by bis(3,5-dibromosalicyl)fumarate. D, hemoglobin A modified by bis(3,5-dibromosalicyl)mesaconate. All reactions were carried out at 37°C with oxyhemoglobin (1 mM) and 1 mM of the compound in 0.2 M bis-Tris buffer, pH 7.2, for 2 h.

TABLE IV
Reactivity of bis(3,5-dibromosalicyl)fumarate and bis(3,5-dibromosalicyl)succinate with intracellular hemoglobin

| Compound          | Hemoglobin | Red cells modified with DIDS | Red cells | DIDS% |
|-------------------|------------|------------------------------|-----------|-------|
| Bis(3,5-dibromosalicyl)succinate | 45         | 15                           | 15        |       |
| Bis(3,5-dibromosalicyl)fumarate  | 75         | 5                            | 5         |       |

*All reactions were carried out in bis-Tris buffer, pH 7.2, for 2 h at 37°C with the concentration of the cross-linking agent equal to 1 mM. For cell-free reactions the concentration of hemoglobin was also 1 mM. The reactions with whole cells were carried out with a 20% erythrocyte suspension.

*Erythrocytes were first treated with DIDS to block the anion channel.

in Fig. 2, the more labile fumarate diester is hydrolyzed almost quantitatively at the outer surface of the red cell membrane before transport into the cell can occur. In these experiments, a 20% suspension of erythrocytes was incubated with the cross-linking reagent at an initial concentration of 1 mM, equivalent to the total concentration of hemoglobin. At various times aliquots were removed and the erythrocytes sedimented. The concentrations of the remaining cross-linking agent and the hydrolysis products were then determined by high-pressure liquid chromatography using a reverse phase C_{18} column. The disappearance of bis(3,5-dibromosalicyl)fumarate is first-order through at least 4 half-lives. The rate constant for this reaction, $1.9 \times 10^{-2}$ min$^{-1}$, is 5 times the pseudo first-order rate constant for the spontaneous hydrolysis of the ester under the same conditions. Hydrolysis stops at the monoester which is detected extracellularly in nearly equivalent amounts. The monoester is much less labile to hydrolysis due to replacement of the second ester group with a carboxylate anion which is no longer electron-withdrawing. The monoester is formed almost quantitatively by
alkaline hydrolysis of the diester and was actually synthesized in this manner (see "Experimental Procedures"). Studies using the purified compound showed that transport of the monoester into the red cell occurs to less than 10% within 2 h. The second hydrolysis product, 3,5-dibromosalicylic acid, is present extracellularly in much lower concentrations due to transport into the erythrocyte. After 2 h, the calculated concentration of 3,5-dibromosalicylic acid within the red cell is 6 times the extracellular concentration. Transport occurs against a concentration gradient due to binding of the compound to hemoglobin, as has been shown recently for the transport of pyridoxal into the erythrocyte (37).

The hydrolysis of bis(3,5-dibromosalicyl) fumarate by the red cell is clearly catalytic. If the compound is added again to the erythrocyte suspension at a concentration of 1 mM after 3 h (greater than 5 half-lives), the rate of hydrolysis is the same as in the original sample (data not shown). The hydrolysis of the ester is not due to the acetylcholinesterase associated with the outer surface of the erythrocyte membrane. This treatment decreased the activity of the enzyme by more than 90% as measured by the hydrolysis of acetylthiocholine (44). Appearance of mono-(3,5-dibromosalicyl)fumarate, appearance of 3,5-dibromosalicylic acid. Reactions were followed in the presence of a 20% red cell suspension in 20 mM sodium phosphate buffer, pH 7.2, with 0.125 M NaCl at 37 °C. At various times aliquots were taken from the reaction mixture and immediately centrifuged, and the extracellular concentration of bis(3,5-dibromosalicyl)fumarate and the two hydrolysis products was measured by high pressure liquid chromatography with a reverse phase C18 column.

The addition of alkyl substituents onto the fumaryl group might be used to block the hydrolysis of the ester catalyzed by the red cell. As shown in Table V, the introduction of a single methyl group onto the cross-link bridge decreases the reactivity of the ester by 10-fold, as measured by the rate of aminolysis with propylamine, and allows the reagent to be transported across the red cell membrane and very effectively modify intracellular hemoglobin. The two-dimensional gel electrophoresis pattern of hemoglobin, modified with the methylfumurate derivative, bis(3,5-dibromosalicyl)mesaconate, is identical to that for the original fumurate diester (see Fig. 1D). The kinetics of transport of the compound into the red cell are first-order (Fig. 3), consistent with passive diffusion. The observed first-order rate constant for disappearance of the compound, 3.2 × 10⁻² min⁻¹, is the sum of the rate constants for hydrolysis and transport. Based on the amount of the monoester formed, more than 90% of the reaction must be due to transport. Since the ratio of hydrolysis to transport is at least 10:1 in the case of the fumarate diester (see Fig. 2), the rate constant for transport of bis(3,5-dibromosalicyl)fumarate must be ≤ 2.0 × 10⁻³ min⁻¹. This is consistent with the value observed for the succinate diester (see Table V). By the addition of a single methyl group, the rate of

### Table V

| Transport of fumaryl and succinyl diesters across the red cell membrane | Cross-linking | Red cell catalyzed hydrolysis rate¹ |
|---------------------------|------------|-------------------------------|
| Hemo(3,5-dibromosalicyl) | %          | Min⁻¹ min⁻¹                   |
| succinate                | 45         | 15                            | 0.22          | 4.4 × 10⁻³ |
| fumarate               | 75         | 5                             | 17.4          | 1.9 × 10⁻² |
| Bis(3,5-dibromosalicyl)mesaconate (2-methyl-fumarate) | 65 | 40                           | 1.76          | 3.2 × 10⁻² |

¹ Reaction conditions were the same as in Table IV.
² Determined directly from the rate of disappearance of the compound in a 20% red cell suspension as described in Fig. 2. The observed rate constant is the sum of the rate constants for hydrolysis and transport. For bis(3,5-dibromosalicyl)fumarate this value is almost entirely due to hydrolysis (see Fig. 2); for the other two reagents it is predominantly due to transport.

![Fig. 2. Hydrolysis of bis(3,5-dibromosalicyl)fumarate catalyzed by the red cell.](image)

![Fig. 3. First-order plot for the disappearance of bis(3,5-dibromosalicyl)mesaconate in the presence of a 20% red cell suspension.](image)
transport of bis(3,5-dibromosalicyl)mesaconate is increased by more than 15-fold. That the added methyl group does significantly increase the overall hydrophobicity of the molecule was evident from the chromatographic behavior of the compounds. The retention time of bis(3,5-dibromosalicyl)mesaconate on the C18 column was increased from 6.9 min observed for bis(3,5-dibromosalicyl)fumarate and -succinate compounds. The retention time of bis(3,5-dibromosalicyl)succinate was evident from the chromatographic behavior of the significantly increase the overall hydrophobicity of the molecule by more than 15-fold. That the added methyl group does not permeate to the red cell membrane (see Table VI). In contrast the n-butylfumarate derivative is readily taken up by the erythrocyte and reacts with intracellular hemoglobin. Since there is little structural requirement for transport by passive diffusion, we would in general expect that the effect of hydrophobic substituents attached to the cross-link bridge and on the leaving group to be additive.

The Effect of Larger Alkyl Substituents Attached to the Cross-link Bridge on the Solubility of Deoxyhemoglobin S—As shown in Table III, the solubility of deoxyhemoglobin S cross-linked by bis(5-bromosalicyl)-2-n-butylfumarate is increased almost 10% above that for the original succinate and fumarate derivatives. The increase in the solubility of deoxyhemoglobin S due to carbamylation of the β NH2 termini by cyanate, ~23% (38), appears to be due to the displacement of the amino-terminal tripeptide and the A helix of the β chains away from the central cavity of the tetramer which directly perturbs the mutation site at Val 6 (39). Steric effects of the butyl group placed within the central cavity on the cross-link bridge may cause a similar change in the structure. X-ray studies are in progress to test this hypothesis. It may be possible to further exploit this effect by the attachment of even larger alkyl groups onto the cross-link bridge and on the leaving group to be additive.

Attachment of Larger Hydrophobic Substituents to the Leaving Group—The correlation observed between the reactivity of the cross-linking agents with hemoglobin and the hydrophobicity of the substituent attached on the leaving group to the ester led us to examine derivatives having larger apolar groups in this position. As shown in Table VI the attachment of phenyl derivatives to the salicylic acid group in compounds 23 and 24 greatly enhances the reactivity toward hemoglobin and also the transport of the cross-linking agent into the red cell. The specific increase in reactivity toward hemoglobin is much greater than with bromine attached to the leaving group. The second-order rate constants for the reaction of bis(5-bromosalicyl)mesaconate and bis(5-(2',4'-difluorophenyl)-salicyl)mesaconate (24) with propylamine are 1.3 M⁻¹ min⁻¹ and 2.8 × 10⁻² M⁻¹ min⁻¹, respectively. Although these two compounds are equally reactive toward hemoglobin, the inherent reactivity of 24 is decreased by nearly 50-fold. As in the case of the butylfumarate derivative (22), a second hydrophobic substituent ortho to the ester (at the 3-position of the salicylic acid ring) is no longer required for diffusion across the red cell membrane.

The leaving group in 24,

![Diagram](image)

is the drug diflunisal used as a long acting analgesic ant-inflammatory agent. This compound can be given in doses of up to 1–2 g/day and has relatively low toxicity. The pharmacological properties of a large number of analogs of this reagent have also been studied (40, 41). These derivatives are of obvious interest in the design of new anti-sickling compounds. Since the salicylic acid group is released in the reaction of the cross-linking agent with hemoglobin, this compound must also be clinically acceptable.

Pharmacological Studies—In previous studies of anti-sickling agents red cells have invariably been treated with relatively high concentrations of the drug. Even with only a 20% erythrocyte suspension, the concentration of the compound must be at least 1 mM just to be stoichiometric with hemoglobin. This high concentration in effect provides the loading dose. For compounds which bind strongly to hemoglobin, a much lower free concentration of the drug would be required. The concentration needed is determined simply by the association constant. This is true for both noncovalent reagents, which bind reversibly, and affinity reagents, which after binding react irreversibly with the protein. In the latter case, the free intracellular concentration of the drug will determine the rate of modification. In the experiments shown

### Table VI

| Compound                  | Cross-linking                   | Reaction conditions were the same as in Table IV. |
|---------------------------|---------------------------------|--------------------------------------------------|
|                           | Hemoglobin % | Red cell |                                            |
| Bis(5-bromosalicyl)     | 15         | 0        |                                           |
| succinate (11)          |            |          |                                            |
| Bis(5-bromosalicyl)     | 28         | 0        |                                           |
| fumarate (20)           |            |          |                                            |
| Bis(5-bromosalicyl)     | 20         | 0        |                                           |
| mesaconate (21)         |            |          |                                            |
| Bis(5-bromosalicyl)     | 19         | 16       |                                           |
| 2-n-butylfumarate (22)  |            |          |                                            |
| Bis(5-phenyl)salicyl)   | 16         | 10       |                                           |
| mesaconate (23)         |            |          |                                            |
| Bis[5-(2',4'-difluorophenyl)-salicyl]mesaconate (24) | 20 | 14 | |
in Fig. 4 erythrocytes were modified with bis(3,5-dibromosalicyl)mesaconate by repeatedly resuspending the cells in a fresh solution of the compound every 2 h (more than 5 half-lives for the rate of transport into the red cell). High levels of modification were readily achieved at steady-state concentrations of the drug as low as 100 μM. In vivo, the free concentration of the drug established by the maintenance dose will determine the toxicity. The initial loading dose would be given over an extended period of time as in the experiments in Fig. 4, rather than as a single bolus.

The viability of red cells modified with bis(3,5-dibromosalicyl)mesaconate in vitro was studied in rabbits. The half-life of red cells treated with 1 mM of the compound as a single dose for 2 h was found to be decreased by more than 40% (see Table VII). In contrast, cells treated 4 times with 0.25 mM of the reagent have almost normal viability when corrected for the damage that occurs simply in repeatedly centrifuging and resuspending the cells in the buffer alone. In this case there is actually a greater level of modification of hemoglobin as well.

As is true for most drugs, the compounds that we are studying have both polar (or charged) and hydrophobic groups under the conditions indicated. All reactions were carried out in 20 mM sodium phosphate buffer, pH 7.2, with 0.125 M NaCl and 10 mM glucose at 37 °C. Each incubation was for 2 h. The half-time is defined in these studies as the time required for 50% of the radioactivity to disappear beginning 1 day after reintroduction of the cells. The values shown are the average of three separate experiments.

| Treatment                     | Half-life | Modification |
|-------------------------------|-----------|--------------|
| Buffer alone (2 h)            | 16        | %            |
| 1 mM Bis(3,5-dibromosalicyl)  | 9         | 30           |
| mesaconate (1 dose)           |           |              |
| Buffer, 4 times               | 13        |              |
| 0.25 mM Bis(3,5-dibromosalicyl)mesaconate, 4 times | 11 | 40 |

CONCLUSION

There remain two principal points for further modifying the structure of the cross-linking agents, i.e. the attachment of alternate substituents onto the cross-link bridge and varying the nature of the hydrophobic substituent attached to the leaving group. In designing new reagents these two structural features may be varied independently. As shown in Fig. 2, the reaction of bis(3,5-dibromosalicyl)fumarate with intracellular hemoglobin is limited by competing hydrolysis of the reagent catalyzed at the outer surface of the red cell membrane. The attachment of simple alkyl groups onto the cross-link bridge decreases the liability of the ester due to steric effects and allows the drug to reach its target site within the red cell. Negatively charged or polar groups may also be introduced in this position. Interactions between these groups and the cluster of positively charged residues within the DPG binding site should increase the affinity for the reagent. For these more polar derivatives larger hydrophobic substituents attached to the salicylic acid group may be essential for transport across the red cell membrane. Phenyl derivatives as in the analgesic diflunisal should be particularly useful for this purpose as these compounds are likely to be clinically acceptable.

Of the compounds studied thus far, bis(3,5-dibromosalicyl)mesaconate is the most effective in modifying intracellular hemoglobin and may be sufficiently specific to be used extracorporeally. At concentrations between 100 and 200 μM there would probably be little deleterious effect on the treated cells. A large reservoir of the compound or a continuous perfusion system would be needed to maintain this fixed concentration of the drug during the reaction.

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