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Diaspirins That Cross-Link β Chains of Hemoglobin: Bis(3,5-dibromosalicyl) Succinate and Bis(3,5-dibromosalicyl) Fumarate†

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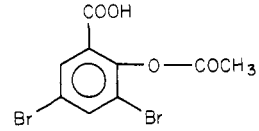
ABSTRACT: Two double-headed aspirins, bis(3,5-dibromosalicyl) succinate and bis(3,5-dibromosalicyl) fumarate, have been found to be potent acylating agents of intracellular hemoglobin (A or S) in vitro. Furthermore, each of these reagents cross-links β chains of hemoglobin, probably at the β cleft. The modified hemoglobins show increased oxygen affinities and reduced gelation or sickling tendencies.

Under suitable conditions, molecules of hemoglobin S interact with each other to build up fibrous aggregates that are capable of distorting an erythrocyte into the characteristic deformed shape associated with sickle cell syndrome. From a molecular viewpoint, two different general approaches are available to counteract sickling. One can attempt to modify HbS[1] at sites in the contact interfaces of the protein molecules in the insoluble fibril so that intermolecular interactions are obstructed directly. Alternatively, one can perturb the conformation of HbS macromolecules so that potentially interacting groups at the interface are pulled out of register and their interactions are weakened indirectly.

X-ray diffraction (Perutz, 1969, 1970, 1972) has shown that the geometry of the tetrameric form of deoxyhemoglobin is substantially different from that of the oxy form. For HbS, it is the deoxy quaternary ensemble that assembles into the fibrous gel. The deoxy conformation has the spatial arrangement of surface groups that are in register to link the macromolecules together to form an extended fibril. In contrast, in the oxy conformation corresponding interactions do not exist. If one could shift the conformational equilibrium toward the oxy form, or better yet if one could covalently clamp HbS in the oxy conformation, sickling tendencies should be reduced. If reagents with this potential were pharmacologically acceptable, they would be promising for clinical trial.

It has been demonstrated previously (Klotz & Tam, 1973; de Furia et al., 1973; Shamsuddin et al., 1974; Bridges et al., 1975) that aspirin acetylates hemoglobin at physiological pH values. Furthermore, it has also been shown (Zaugg et al., 1975) that diaspirin, bis(*o*-carboxyphenyl) succinate, cross-links subunits of hemoglobin. Unfortunately, the reactivities of these salicylates are not high, 0.02 M concentrations being needed to obtain appreciable transacylation. On the other hand, marked increases in the acylating activity of aspirins have been obtained by modification of the salicylate leaving group (Walder et al., 1977). In particular, 2-acetyl-3,5-dibromosalicylic acid (I) has been found to be a potent acylating



I

agent of intracellular hemoglobin even at 5 mM concentration with acetylation occurring at relatively specific sites on the protein. On this basis, it seemed promising to prepare bis(3,5-dibromosalicyl) diesters as more effective cross-linking agents. This paper describes the behavior of two such alternative diaspirins, bis(3,5-dibromosalicyl) succinate (II) and bis(3,5-dibromosalicyl) fumarate (III).

Materials and Methods

Bis(3,5-dibromosalicyl) succinate was prepared by the procedure used for the corresponding salicylate diesters (Zaugg et al., 1975). One mole of succinyl chloride was added dropwise to 2 mol of 3,5-dibromosalicylic acid and 4 mol of *N,N*-dimethylaniline dissolved in benzene. The system was stirred at room temperature overnight. Ice-cold acidified water was mixed with the benzene solution, and ethyl acetate was added. The aqueous layer was removed. The organic phase

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[1] Abbreviations used: HbS, sickle cell hemoglobin; HbA, normal adult hemoglobin; p_{O_2}, pressure of oxygen gas in equilibrium with hemoglobin; $NaDodSO_4$, sodium dodecyl sulfate.

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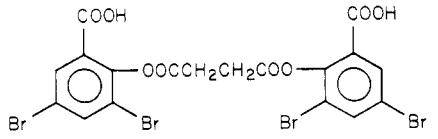
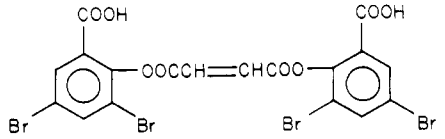


II



III

was extracted several times with water and then dried with Na_2SO_4. The solvents were removed in vacuo. The blue residue was washed several times with benzene to produce a white solid. This product was recrystallized twice from ethanol, mp 195–196 °C. The NMR spectrum was consistent with the presumed structure and revealed the presence of one molecule of ethanol of crystallization. Anal. Calcd for $C_{18}H_{10}O_8Br_4 \cdot C_2H_6O$: C, 33.36; H, 2.24. Found: C, 33.49; H, 2.34.

Bis(3,5-dibromosalicyl) fumarate was prepared by a corresponding procedure similar to that for the succinate diester. The product was recrystallized twice from ethanol. Anal. Calcd for $C_{18}H_8O_8Br_4 \cdot C_2H_6O$: C, 33.46; H, 1.97. Found: C, 33.16; H, 1.80. In a second synthesis, the product was recrystallized twice from acetone–ethyl acetate, mp 226–227 °C. This material showed an NMR spectrum consistent with the presumed structure and revealed no solvent of crystallization.

Sickle cell blood was obtained from Dr. George R. Honig of Children's Memorial Hospital, Chicago, IL, as residual blood from homozygous SS individuals. We are indebted to Dr. Honig for his continued assistance. Washing of erythrocytes and the preparation of buffered erythrocyte suspensions and cell-free hemoglobin solutions were conducted as described previously (Zaugg et al., 1977).

Chemical modifications of hemoglobin were carried out with 20% (v/v) erythrocyte suspensions in isotonic phosphate buffer, pH 7.2, and in 6 g/dL solutions of cell-free hemoglobin in 0.05 M sodium phosphate and 0.01 M sodium cyanide, pH 7.2. These single-dose experiments were supplemented by multiple-dose tests in which erythrocyte suspensions were exposed to successive doses of compound, each fresh exposure being preceded by removal of the previous dose by centrifugation. All incubations took place for 2 h at 37 °C in a water bath shaker. Reactions were terminated by rapid freezing in a dry ice–methanol bath.

The extents of acylation of hemoglobin were assessed by isoelectric focusing in polyacrylamide gels as described previously (Zaugg et al., 1977). Acylated species appeared as focused bands anodal to unmodified HbA. After being electrofocused, the gels were removed from the tubes and fixed in 15 g/dL trichloroacetic acid to prevent diffusion of the focused bands. The extents of acylation were quantitated by integration of peaks in densitometric scans of the gels.

Each of the bis(3,5-dibromosalicyl) diesters was mixed with 6 g/dL HbA to investigate the formation of chemical cross-links. Incubations were carried out in 0.05 M phosphate and 0.01 M cyanide or in 0.05 M Bistris-HCl, pH 7.2, for 2 h at 37 °C. The occurrence of cross-links between subunits of the hemoglobin tetramer was detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to established procedures (Shapiro et al., 1967; Weber & Osborn, 1969). Hemoglobin solutions were diluted to a concentration of 4 mg/mL in 0.01 M sodium phosphate, pH 7.2, which contained 1 g/dL sodium dodecyl sulfate and 1 mg/dL 2-mercaptoethanol. Protein solutions were then placed in a boiling water bath for several minutes to ensure complete denaturation. Gel tubes (85 × 5 mm, i.d.) contained 10 g/dL acrylamide, 0.4 g/dL bis(acrylamide), and 0.1 g/dL sodium dodecyl sulfate in 0.1 M sodium phosphate, pH 7.2. Gel polymerization was accomplished by addition of 0.5 mg/mL ammonium persulfate and 0.3 μg/mL *N,N,N',N'*-tetramethylethylenediamine. Hemoglobin solutions (5–10 μg in 0.02 g/dL bromophenol blue, 0.1 g/dL sodium dodecyl sulfate, 10 g/dL glycerol, and 0.1 M sodium phosphate, pH 7.2) were electrophoresed at 6 mA/gel for 5 h by using as electrolyte a solution of 0.1 M sodium phosphate, pH 7.2, containing 0.1 g/dL sodium dodecyl sulfate. After electrophoresis, the gels were stained for several hours in water–methanol–acetic acid (9:9:2) containing 0.25 g/dL Coomassie blue. Destaining was accomplished in a Bio-Rad, Model 172A, diffusion destaining apparatus that contained water–methanol–acetic acid (17:2:1). The extents of cross-linking were quantitated by integration of peaks in densitometric scans of the stained gels.

Oxygen-binding curves were measured as described previously (Zaugg et al., 1977) with the exception that erythrocytes were suspended in isotonic phosphate buffer (0.123 M sodium phosphate, pH 7.2) that contained 5 mM glucose rather than in buffer alone. Glucose served to prevent the intracellular depletion of 2,3-diphosphoglycerate during lengthy incubations.

Investigations of the reversibility of the enhanced oxygen affinity of treated erythrocytes were conducted by resuspending 1 mL of packed red cells in several 30-mL portions of reagent-free buffer. The influence of this "washing" procedure on the oxygen affinity was evaluated by measuring the percent of oxyhemoglobin at a fixed p_{O_2} value (22–23 mmHg). This partial pressure provided O_2 saturations in the midrange of the oxygen uptake curves.

Tests for antisickling activity were conducted as described previously (Zaugg et al., 1977).

Minimum gelling concentrations (MGCs) were determined according to the procedure of Bookchin & Nagel (1971). Washed sickle erythrocytes in isotonic phosphate buffer, pH 7.2, were exposed to acylating agent for 2 h at 37 °C, after which the red cells were packed by centrifugation. Packed erythrocytes were then freeze-fractured in a dry ice–methanol bath, thawed, mixed with 0.8 volume of buffer, and then freeze-thawed a second time. This procedure produced a hemolysate containing 16–18 g/dL HbS. Portions of this hemolysate (0.8–1.0 mL) were placed in 10-mL flasks and exposed to a stream of partially humidified N_2 gas for at least 1 h to ensure complete deoxygenation. Increasing the gas flow rate caused a gradual dehydration of the sample until the gelling point was attained. The sample was then reliquified by cooling and exposure to air, after which its concentration was measured as the cyanomet derivative by using reagents obtained from Hycel.

Results

It has been found previously (Zaugg et al., 1975) that disalicyl succinate, diaspirin, acylates hemoglobin more effectively than does aspirin. Furthermore, 2-acetyl-3,5-dibromosalicylic acid, dibromoaspirin, is much more reactive toward hemoglobin than is the nonbrominated acetylsalicylic acid. It was not surprising, therefore, that bis(3,5-dibromosalicyl) succinate at 1 mM concentration was found to react almost quantitatively with hemoglobin. Thus, a 6% solution of oxyhemoglobin A (about 1 mM in concentration)

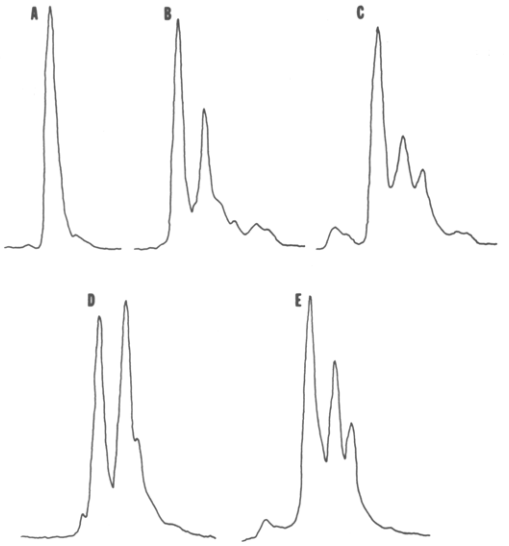


FIGURE 1: Densitometric scans at 546 nm of isoelectrically focused gels containing hemoglobin A modified with disalicyl succinate and with bis(3,5-dibromosalicyl) succinate. A 6% solution of cell-free hemoglobin in 0.05 M phosphate buffer, pH 7.2, was allowed to react with either compound for 2 h at 37 °C. (A) Untreated hemoglobin; (B and C) hemoglobin after reaction with 20 mM disalicyl succinate under fully oxygenated and fully deoxygenated conditions, respectively; (D and E) hemoglobin after reaction with 1 mM bis(3,5-dibromosalicyl) succinate under fully oxygenated and fully deoxygenated conditions, respectively. Anode is at the right in each frame. Unmodified HbA is first large peak at the left in each frame.

Table I: Extent of Modification and Cross-Linking of Cell-Free HbA[a]

	oxy-HbA		deoxy-HbA	
reagent	% modification	% cross-linking	% modification	% cross-linking
disalicyl succinate (20 mM)	35	10	35	0
bis(3,5-dibromosalicyl) succinate (1 mM)	70	40	50	<5

[a] Each reaction was allowed to proceed for 2 h at 37 °C in 0.05 M phosphate buffer, pH 7.2; [Hb] = 6% (~1 mM).

was 70% modified by 1 mM bis(3,5-dibromosalicyl) succinate (Figure 1). In contrast, nonbrominated diaspirin at 20 mM concentration yielded only 40% modification of oxyhemoglobin (Figure 1). Isoelectric focusing of the modified oxyhemoglobins revealed one major modified band. From the nearly 1:1 stoichiometry of the reaction with bis(3,5-dibromosalicyl) succinate, the principal isoelectric band must be due largely to singly labeled protein, but this need not be a unique species of modified hemoglobin.

Electrophoresis in the presence of sodium dodecyl sulfate was used to identify cross-linked hemoglobin chains. Figure 2 shows sodium dodecyl sulfate–polyacrylamide gels for untreated HbA and for HbA modified with 20 mM disalicyl succinate or with 1 mM bis(3,5-dibromosalicyl) succinate under oxygenated conditions. The modified hemoglobins show a new component (arrow) of molecular weight 32 000 (established by comparison with marker proteins). Since this value is twice the molecular weight of isolated hemoglobin chains, the new band must represent a dimeric species. In the gel, α and β monomeric chains are separated. Only the β chains are diminished after modification by the diesters. Hence the cross-linked species must represent a β_2 dimer.

Extents of cross-linking of oxy-HbA by disalicyl succinate and by bis(3,5-dibromosalicyl) succinate are listed in Table

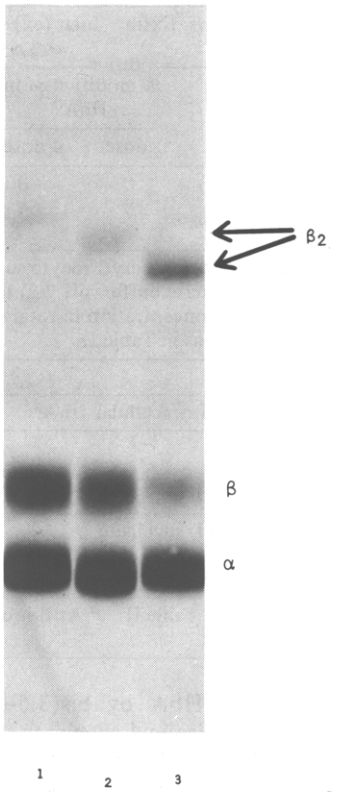


FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gels demonstrating cross-linking of β chains of hemoglobin A by disalicyl succinate. (1) Unmodified HbA; in addition to α and β chains, two other red cell proteins, in much lower abundance than hemoglobin, can be seen; (2 and 3) HbA treated with disalicyl succinate and bis(3,5-dibromosalicyl) succinate, respectively, corresponding to isoelectric focusing scans (B) and (D) in Figure 1. The cross-linked dimer is marked by arrows. Only the β chains are depleted. Gels were positioned so as to align the α and β chains, but because the gels ran to slightly different extents other bands are a little off register. The anode is at the bottom. Protein bands were stained with Coomassie brilliant blue.

I along with the values for the total extent of modification as measured by isoelectric focusing. For the reaction of 1 mM bis(3,5-dibromosalicyl) succinate with 1 mM oxy-HbA, cross-linking accounts for over 50 percent of the modification. If the cross-linking reaction is due to a singular modification, as seems likely (see below), this reagent shows a substantial degree of specificity in its reaction with hemoglobin. This specificity is particularly striking when one recognizes that there are 24 distinct amino groups per $\alpha\beta$ dimer, almost all of which are on the surface of the protein (A. Arnone, personal communication) and might react with bis(3,5-dibromosalicyl) succinate.

The behavior of bis(3,5-dibromosalicyl) fumarate was very similar to that of the corresponding succinate diester, the former producing even higher extents of modification and crosslinking.

Cross-linking of the β chains may occur by either an intertetrameric or an intratetrameric pathway. To differentiate between these two possibilities, we studied the cross-linking as a function of hemoglobin concentration. The extent of cross-linking was found to be essentially independent of hemoglobin concentration (range studied was 0.1–1 mM) for reaction with 5 mM bis(3,5-dibromosalicyl) succinate. The lack of dependence on hemoglobin concentration is consistent with a unimolecular (intratetrameric) mechanism.

The most probable site of cross-linking of the β chains is across the β cleft. The span of this portal is substantially different in deoxyhemoglobin as compared to oxyhemoglobin.

Table II: Intracellular (int) vs. Extracellular (ex) Modification of HbA

reagent	% modifien of int HbA[a]		% modifien of ex HbA,[b]
	1 dose	4 doses	1 dose
disalicyl succinate (5 mM)	0	0	14
bis(3,5-dibromosalicyl) succinate (1 mM)	18	64	70

[a] Each dose involved exposure of erythrocyte suspensions (20% by volume in 0.123 M phosphate buffer, pH 7.2) to the reagent for 2 h at 37 °C. Hemoglobin concentration in total volume was ~1 mM. [b] Reaction conditions as in Table I.

Table III: Cross-Linking of Intracellular HbA[a]

	concn (mM)	% cross-linking
bis(3,5-dibromosalicyl) succinate	1	10
	2	20
	5[b]	45

[a] Reaction conditions as in Table II. [b] At this concentration some hemolysis was observed.

Cross-linking of deoxy-HbA by bis(3,5-dibromosalicyl) succinate as well as by disalicyl succinate was found to be strikingly reduced compared to the reaction with oxy-HbA (Table I). A possible explanation for this observation is that the binding of 2,3-diphosphoglycerate, which is present during the usual reaction conditions, to deoxy-HbA blocks access to the β cleft. Thus, the diaspirins may be unable to enter the cavity to acylate amino side chains. To examine this possibility, we studied the reaction with deoxy-HbA that had been stripped of 2,3-diphosphoglycerate. Cross-linking of the β chains was still reduced despite the removal of the barrier at the entrance of the cavity. Evidently it is the disposition of the amino groups within the β cleft of deoxy-HbA that is not appropriate for cross-linking with either succinate diester.

Both succinate diesters, nevertheless, do react with deoxy-HbA. The overall extent of modification is comparable to that for reaction with oxy-HbA (Table I), but the pattern of species in isoelectric focusing gels is markedly different (Figure 1). In particular, there is a greater number of prominent bands, indicative of lesser specificity in the reaction.

As described previously (Zaugg et al., 1975), disalicyl succinate does not react with intracellular hemoglobin, presumably because it does not cross the red blood cell membrane. In contrast, bis(3,5-dibromosalicyl) succinate does penetrate the erythrocyte membrane and modify intracellular hemoglobin (Table II). Cross-linking of the β chains of intracellular hemoglobin is also seen (Table III). The lipophilic bromine substituent contributes to the reagent not only an increase in intrinsic reactivity with hemoglobin but also greater membrane permeability. The extent of modification of intracellular hemoglobin by bis(3,5-dibromosalicyl) succinate is less, however, than that observed with extracellular hemoglobin under identical conditions and protein concentration (Table II). Hence, the erythrocyte membrane is not fully permeable to this compound.

Since bis(3,5-dibromosalicyl) succinate reacts with intracellular hemoglobin, studies of its effects on oxygen-binding and sickling properties were undertaken. In Figure 3 are shown oxygen-binding curves for normal erythrocytes treated with bis(3,5-dibromosalicyl) succinate (1 mM × 2 doses; 42% modification of HbA) and with 3,5-dibromosalicylic acid (2 mM × 2 doses). The increase in oxygen affinity of hemoglobin

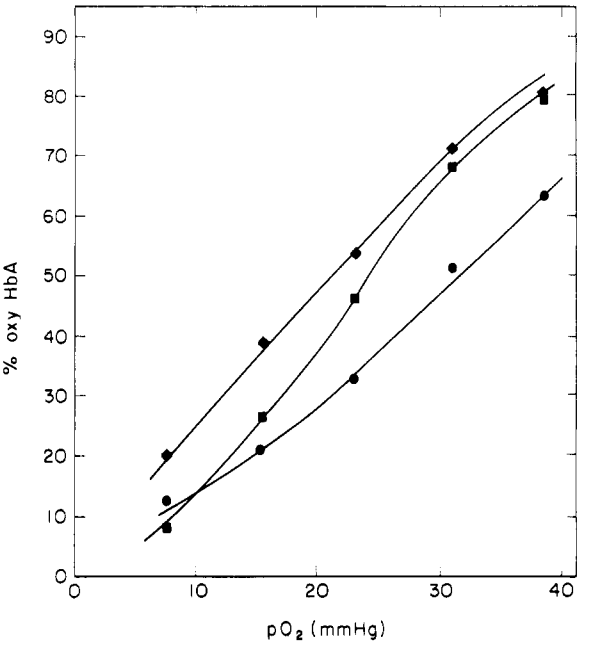


FIGURE 3: Oxygen-binding curves for normal erythrocytes. Erythrocyte suspensions (20% v/v) in isotonic phosphate buffer were untreated (●) or were exposed to 3,5-dibromosalicylic acid, 2 mM × 2 doses (■), or to bis(3,5-dibromosalicyl) succinate, 1 mM × 2 doses (♦). Each dose was allowed to contact Hb for 2 h at 37 °C. Immediately thereafter, oxygen equilibrium curves were measured.

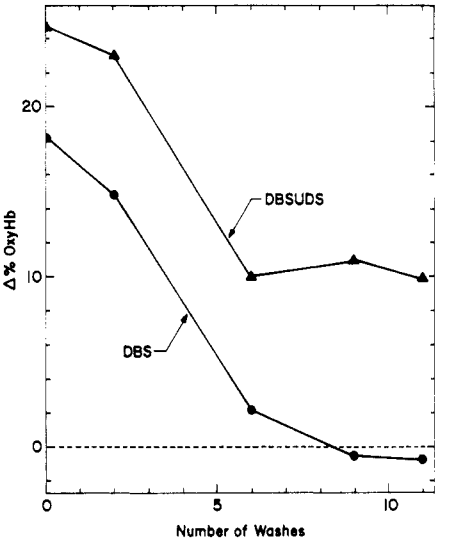


FIGURE 4: Dialysis washing experiment demonstrating the residual effect on oxygen binding due to acylation of hemoglobin A by bis(3,5-dibromosalicyl) succinate. Erythrocyte suspensions (20% v/v) were treated with 3,5-dibromosalicylic acid (DBS), 2 mM × 2 doses (●), or with bis(3,5-dibromosalicyl) succinate (DBSUDS), 1 mM × 2 doses (▲), in isotonic phosphate buffer, pH 7.2, containing 5 mM glucose. After exposure to the reagent, each dose was mixed for 2 h at 37 °C and 1 mL each of treated and of untreated packed cells was washed repeatedly in reagent-free buffer. Abscissa: the number of resuspensions for 10 min each in 30 mL of reagent-free buffer. Ordinate: the difference in percent oxy-Hb between treated and untreated erythrocyte suspensions after equilibration with a gas mixture containing 97% N_2 and 3% O_2 (p_{O_2} = 23 mmHg).

treated with 1 mM bis(3,5-dibromosalicyl) succinate[2] is substantially greater than that with 2 mM dibromoaspirin. It

[2] The sample of bis(3,5-dibromosalicyl) succinate that was used contained one molecule of ethanol of crystallization. Control experiments showed that at the concentrations of the reagent tested (1–5 mM) ethanol had no effect on the oxygen-binding or sickling properties of hemoglobin.

Table IV: Increase in O_2 Affinity Due to Acylation of Intracellular HbA

reagent	concn (mM)	% modification of intracellular HbA	% decrease in P_{50} after dialysis[a]
2-acetyl-3,5-dibromosalicylic acid	5	75	8
bis(3,5-dibromosalicyl) succinate	1 (2×)	42	18

[a] Cells were washed in reagent-free buffer until there was no residual effect on oxygen binding in a parallel treatment with 3,5-dibromosalicylic acid.

has been shown recently (Walder et al., unpublished experiments) that 3,5-dibromosalicylic acid, which is released in the transacylation reaction, is bound noncovalently by hemoglobin and thereby contributes in itself to the modulation of oxygen affinity. With bis(3,5-dibromosalicyl) succinate, however, the increase in oxygen affinity is far greater than could be accounted for by all the 3,5-dibromosalicylate that could be released on aminolysis. Thus, acylation per se by the diester must bring about an increase in oxygen affinity.

Further evidence for this was provided by studies of oxygen binding of modified erythrocytes after the cells were washed in reagent-free buffer. As is apparent in Figure 4, washing the cells completely abolishes the effect on oyxgen binding of 3,5-dibromosalicylic acid, a noncovalent modifying reagent. Clearly, washing removes the noncovalent modifier. However, after treatment of erythrocytes with bis(3,5-dibromosalicyl) succinate, there remains a substantial increase in oxygen affinity that is unaffected by the dialysis procedure. This residual effect must be due to acylation of hemoglobin. It is much greater than reported recently (Walder et al., unpublished experiments) for acetylation of hemoglobin by 2-acetyl-3,5-dibromosalicylic acid (Table IV).

The reaction of disalicyl succinate and of bis(3,5-dibromosalicyl) succinate with oxygenated hemoglobin S was investigated. Isoelectric focusing revealed the same pattern of modified species as had been observed with oxy-HbA treated with these reagents. Moreover, the total extent of modification as well as the fraction of cross-linking of the β chains was not significantly different from those values for HbA. As with normal red blood cells, only the dibromodiester was able to cross the sickle erythrocyte membrane to modify and cross-link intracellular HbS.

Oxygen affinity of intracellular HbS was increased by modification of oxygenated sickle erythrocytes with bis-(3,5-dibromosalicyl) succinate to an extent similar to that with normal red blood cells. As with normal erythrocytes, both acylation of the hemoglobin and noncovalent binding of 3,5-dibromosalicylic acid to the protein contribute to the increase in oxygen affinity.

Erythrocyte sickling was inhibited by bis(3,5-dibromosalicyl) succinate[2] but not to any greater extent than could be accounted for by 3,5-dibromosalicylic acid released upon the transacylation reaction with hemoglobin. The effect of 3,5-dibromosalicylic acid on red cell sickling has been described previously (Walder et al., unpublished experiments). With dialysis 3,5-dibromosalicylic acid can be removed (Figure 4). However, such extensive washing causes abnormal changes in the morphology of sickle erythrocytes which make it difficult to determine the effects of acylation per se on sickling by in vitro assay. Nevertheless, gelation experiments, with one HbS blood sample, do indicate that acylation inhibits the gelation of deoxy-HbS (Table V).

Table V: Minimum Gelling Concentration of Hemoglobin Extracted from Sickle Erythrocytes[a,b]

minimum gelling concentration (g/dL)		
untreated	3,5-dibromosalicylic acid (2 mM × 2)	bis(3,5-dibromosalicyl) succinate[c] (1 mM × 2)
21.9	25.1	28.3

[a] These results were obtained with blood from only one individual and must therefore be regarded as preliminary. [b] Sickle erythrocyte suspensions were modified as described in Table II, and HbS was extracted from the lysed cells. [c] Approximately 35% of the hemoglobin was modified, 20% cross-linked.

Discussion

Both brominated diesters described here and the nonbrominated diaspirin examined previously (Zaugg et al., 1975) are markedly more effective in the acylation of hemoglobin than are their corresponding monoesters. This increased reactivity may arise from several structural features. The diesters containing two rings provide additional components for apolar interactions to strengthen ligand binding by the protein. Further electrostatic assistance in the stabilization of bonding may come from the doubled anionic charge on the diesters. Additional contributions to apolar interactions are also provided by the bromine substituents, as has been directly demonstrated by equilibrium dialysis experiments (Walder et al., unpublished experiments). The double-headed aspirins, particularly the fumarate, may also be more susceptible to aminolysis because of the inductive effects of the second ester group. All of these elements probably contribute to the superior abilities of the bromo diesters to modify hemoglobins.

The addition of bromine substituents also endows the diesters with the capacity to penetrate the erythrocyte membrane, a property denied to the corresponding nonbrominated disalicyl diesters. The dibromodiaspirins thus have the potential of producing their modifications of hemoglobin structure and behavior in vivo.

Particularly attractive about the diesters is their ability to cross-link β subunits of hemoglobin. The $NaDodSO_4$ gels revealing dimeric species of 32 000 molecular weight (Figure 2) show depletion of only the β monomers after hemoglobin has been treated with bis(3,5-dibromosalicyl) succinate. Similar exclusivity has been found in cross-linking by disalicyl succinate (Zaugg, 1978). There is no evidence of covalent linkages between α subunits. In view of the double anionic charge on the diesters, they should be attracted electrostatically to regions of high cationic charge in (tetrameric) hemoglobin, such as is in the β cleft (Arnone, 1972).

The increase in oxygen affinity of HbA due to acylation by bis(3,5-dibromosalicyl) succinate may result from either the cross-linking of the β chains or the non-cross-linking modifications at other sites. The observation that cross-linking occurs far more readily in the oxygenated state suggests that cross-linking favors the oxy conformation of hemoglobin relative to the deoxy conformation and hence increases the oxygen affinity. Moreover, if cross-linking does indeed occur across the β cleft, this should block the binding of 2,3-diphosphoglycerate to deoxyhemoglobin and on this basis increase the oxygen affinity of intracellular hemoglobin. To determine unambiguously the effect of cross-linking of the

chains on oxygen binding, it will be necessary to study the purified cross-linked species.

Within the β cleft of deoxyhemoglobin the distances between pairs of amino groups on opposite β chains are as follows (Arnone, 1972; Benesch et al., 1975): Lys-82···Lys-82, 8.1 Å; Lys-82···Val-1, 11 Å; Val-1···Val-1, 18 Å. In oxyhemoglobin, the first two spans are shorter. From molecular models we estimate the following distances between N atoms attached to opposite ends of fully extended dicarboxylic acids: $-(O{=})CCH{=}CHC(=O)-$, 6.8 Å; $-(O{=})C(CH_2)_2C(=O)-$, 6.8 Å; $-(O{=})C(CH_2)_4C(=O)-$, 9.2 Å. Comparison of these values with the distances between amino groups across the β cleft rules out the possibility of a four-carbon bridge between the valine N termini. Of the other two potential sites for cross-linking, a Lys-82 → Lys-82 bridge would be favored since its span is the shorter one.

With the disalicyl diesters, it has also been found (Zaugg, 1978) that longer bridges, for example, with adipate and suberate linkages, are *not* formed when oxyhemoglobin is treated with the respective diaspirins.[3] Evidently the $-NH_2$ sites most susceptible to cross-linking are spanned best by a four-carbon bridge. Clearly, the β portal of hemoglobin is sharply selective in the structures that have steric access to this cavity. This selectivity is an attractive feature in the potential use of diaspirins as antisickling agents.

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[3] Furthermore, in the present study, bis(3,5-dibromosalicyl) sebacate, with a C_{10} bridge, was found not to cross-link either oxyhemoglobin or deoxyhemoglobin.

Purification of the Messenger Ribonucleic Acid for the Lipoprotein of the *Escherichia coli* Outer Membrane†

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ABSTRACT: The mRNA for the lipoprotein of the *Escherichia coli* outer membrane has been purified to 85% homogeneity. The purification procedure involved phenol extraction, NaCl extraction, gel filtration on Sephadex G-100 and Sephadex G-200, and reversed-phase column chromatography on RPC-5. The purity of the final product was estimated to be 85% by analysis of the ribonuclease T_1 fingerprint of the mRNA. The purified mRNA was able to direct the synthesis of cross-reactive material with antilipoprotein serum in both the *E. coli* and the wheat germ cell-free protein-synthesizing systems. The size of the mRNA was determined to be 8.2 S from its mobility in polyacrylamide–agarose gels. During the purification, two other RNA species, similar in size to the lipoprotein mRNA, were also isolated. Their sizes were determined to be 8.7 and 9.1 S. They both were inactive in an *E. coli* cell-free protein-synthesizing system.

The lipoprotein of the *Escherichia coli* outer membrane is one of the most thoroughly investigated membrane proteins of procaryotic organisms [see a review by DiRienzo et al. (1978)]. Biosynthesis of the lipoprotein in vivo and in vitro has also been investigated [see a review by Inouye (1979)]. The mRNA for the lipoprotein has been shown to be highly stable (Hirashima & Inouye, 1973; Hirashima et al., 1973). Cross-reactive material with antilipoprotein serum was produced in the *E. coli* cell-free protein-synthesizing system (Hirashima et al., 1974), as well as in the wheat germ system (Wang et al., 1976), directed by the purified mRNA. The primary product in the *E. coli* cell-free system was found to be a precursor of the lipoprotein, prolipoprotein, which has 20 additional amino acid residues at the amino-terminal end of the lipoprotein, and the amino acid sequence of the prolipoprotein was determined (Inouye et al., 1977). The in vivo ^{32}P-labeled lipoprotein mRNA has been identified as the mRNA which codes for the lipoprotein (Takeishi et al., 1976), and the nucleotide sequence of the 5′ end of the mRNA has been determined (Pirtle et al., 1978).

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