

Alternative aspirins as antisickling agents: Acetyl-3,5-dibromosalicylic acid

(hemoglobin S/aspirin analogs/sickle cell anemia/protein modification)

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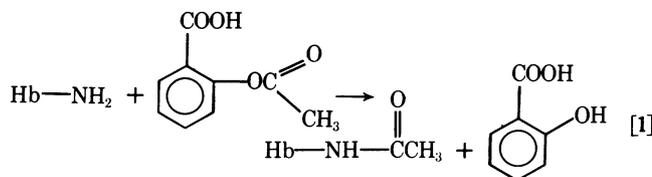
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ABSTRACT Acetyl-3,5-dibromosalicylic acid (dibromoaspirin) is shown to be a potent acylating agent of intracellular hemoglobin *in vitro*. Transfer of the acetyl group of dibromoaspirin to amino groups of hemoglobins A and S seems to occur predominantly at just two or three sites on these proteins. This acetylation produces moderate increases in the oxygen affinities of normal and sickle erythrocytes. Furthermore, treatment of intracellular hemoglobin S with dibromoaspirin directly inhibits erythrocyte sickling. This antisickling effect is paralleled by an increase in the minimum gelling concentration of deoxy hemoglobin S extracted from sickle erythrocytes that had been exposed to low concentrations of dibromoaspirin. These observations suggest that dibromoaspirin might be an effective antisickling agent *in vivo*.

On deoxygenation, hemoglobin S (Hb S) aggregates into helical fibers that precipitate from solution as a gel (1, 2). Within the erythrocyte, these fibers distort the cell into various abnormal shapes, among which is the characteristic sickled form. Although other abnormalities of the erythrocyte have been described in patients having sickle cell anemia (3-5), the aggregation of deoxy Hb S represents the principal pathologic process underlying the disease.

It has occurred to a number of investigators that the aggregation of deoxy Hb S might be inhibited by chemical modification of the protein (6-11). Of the different functional groups in the side chains of this protein, the amino group seems particularly attractive because it can be modified with relatively mild reagents that might be pharmacologically acceptable. One category of such reagents includes compounds such as cyanate (6, 12) and aspirin (8, 13, 14), which carbamylate and acylate hemoglobin, respectively. A second group, aldehydes, form Schiff base adducts with amines of the protein (9, 15-20). Cyanate (6, 12) and a number of aromatic aldehydes (18, 20) have been shown to inhibit erythrocyte sickling *in vitro*. These compounds exert their antisickling effects by increasing the oxygen affinity of Hb S, thereby decreasing the fraction of Hb S in the deoxy form. At fixed percentages of saturation of hemoglobin with oxygen, however, these compounds have no significant influence on sickling.

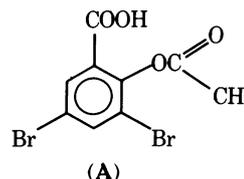
The finding that aspirin acetylates amino groups of intracellular hemoglobin



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and thereby increases its affinity for oxygen suggested to us that aspirin might be a candidate for the treatment of sickle cell disease (8). However, further studies revealed that the extent of acetylation achieved by even high concentrations of the drug was not sufficient to produce a significant inhibition of erythrocyte sickling (13, 21, 22). Aspirin, however, is only one, perhaps 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 (the salicylate) or the acyl function. An investigation of a series of such alternative aspirins therefore was undertaken.

We report here that a compound modified in the leaving moiety, acetyl-3,5-dibromosalicylic acid (A), is a very effective acylating agent of intracellular hemoglobin.



Acetylation appears to occur at relatively specific sites on the protein. The compound inhibits significantly the gelation of cell-free deoxy Hb S and erythrocyte sickling.

EXPERIMENTAL PROCEDURES

Materials. 3,5-Dibromosalicylic acid was obtained from Aldrich Chemical Co. Acetyl-3,5-dibromosalicylic acid (dibromoaspirin) was prepared by the sulfuric acid-catalyzed reaction of 3,5-dibromosalicylic acid with a 2.5-fold excess of acetic anhydride. When the reaction mixture was poured into ice water, the product separated as a white solid: mp 152-153.5° (recrystallized from benzene); lit. 156° (23). Analysis: calculated for $\text{C}_9\text{H}_6\text{Br}_2\text{O}_4$; C, 31.98%, H, 1.79%; found; C, 32.15%, H, 1.65%. Nuclear magnetic resonance: $\delta_{\text{Me}_4\text{Si}}$ (dimethyl- d_6 sulfide) 2.30 (singlet, 3H, CH_3), 8.03 (doublet, 1H, $J = 2.6$ Hz, aromatic), 8.20 (doublet, 1H, $J = 2.6$ Hz, aromatic).

Carrier ampholytes (pH 6-8) for isoelectric focusing were obtained from LKB, acrylamide and bis-acrylamide from Aldrich, and ammonium persulfate and N,N,N',N' -tetramethylethylenediamine (TEMED) from Bio-Rad.

Methods. Whole blood was drawn by venipuncture from healthy adults. EDTA was used as anticoagulant. Sickle cell blood was obtained from the Hematology Department, Cook County Hospital, Chicago, IL, as residual blood from homozygous SS individuals. For studies using 20% (vol/vol) erythrocyte suspensions in isotonic phosphate buffer (0.123 M sodium

Abbreviations: Hb S, sickle cell hemoglobin; MGC, minimum gelling concentration; Hb A, normal adult hemoglobin.

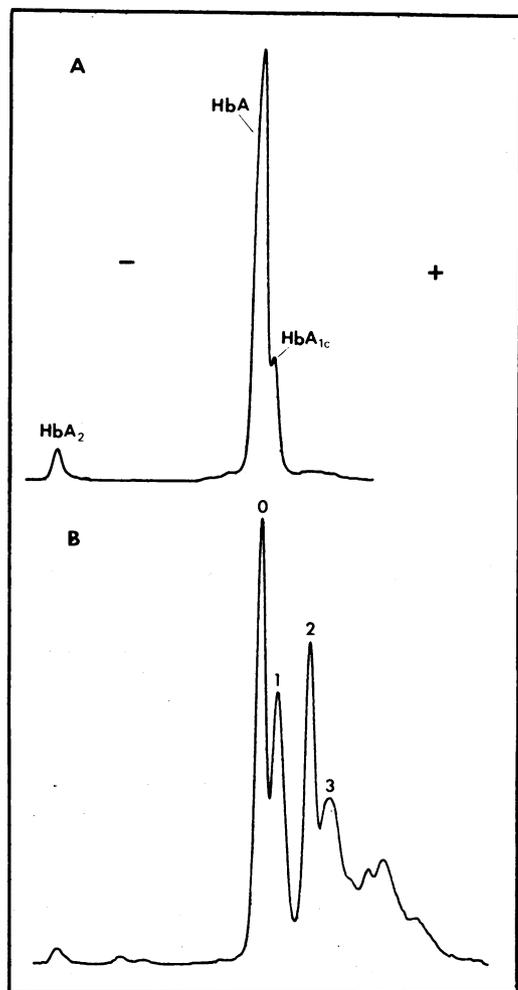


FIG. 1. Densitometric scans at 546 nm of isoelectrically focused gels containing hemoglobin extracted from erythrocytes. (A) Untreated, showing principal hemoglobin components of normal adult erythrocytes. (B) Intracellular hemoglobin after exposure to 5 mM dibromoaspirin for 2 hr at 37° in isotonic phosphate buffer (pH 7.2). Peaks: 0, unmodified Hb A; 1–3, acetylated species of Hb A.

phosphate), pH 7.2, erythrocytes were washed twice in isotonic saline and once in isotonic phosphate buffer.

Reactions of intracellular and cell-free hemoglobin with dibromoaspirin were carried out according to described procedures (20). Additional studies were performed in which treated erythrocytes were centrifuged at hourly intervals and immediately resuspended in fresh buffer containing dibromoaspirin. All reactions were run at 37°.

The chemically modified hemoglobins were examined by gel isoelectric focusing as described (20). The modified species appeared in the gel at positions anodal to unmodified hemoglobin. After electrofocusing, gels were fixed in 15% trichloroacetic acid to prevent diffusion of the sharply focused bands. The extent of modification was quantitated by integration of peaks in densitometric scans of the gels.

Oxygen binding studies and tests *in vitro* for antisickling activity were conducted as described (20). In sickling experiments, 20% suspensions of erythrocytes were first treated with dibromoaspirin in isotonic phosphate buffer. The erythrocytes were then washed once in isotonic phosphate buffer and once in fresh, previously frozen human AB plasma and were finally resuspended in the latter to the original hematocrit value.

Minimum gelling concentrations (MGCs) were determined

according to the procedure of Bookchin and Nagel (24). Measurements were made in parallel on untreated Hb S and on Hb S extracted from erythrocytes after exposure to 5 mM dibromoaspirin for 2 hr at 37° in isotonic phosphate buffer. To ensure complete deoxygenation, samples were exposed to partially humidified N₂ for a minimum of 1 hr. Several determinations were made on each hemoglobin sample. MGC values were reproducible to within 0.5 g/dl.

RESULTS

The reaction of 5 mM dibromoaspirin with a 20% suspension of normal erythrocytes in isotonic phosphate buffer pH 7.2 at 37° for 2 hr led to the modification of 70–80% of the hemoglobin. At these low concentrations, aspirin yields no detectable modification. A densitometric scan of an isoelectrically focused gel on which the hemoglobin species were separated is shown in Fig. 1. The bands appear as a series of doublets. The bands marked 1, 2, and 3 represent approximately 80% of the modified species.

Under the same conditions but with intracellular Hb S, the reaction with 5 mM dibromoaspirin led to 60% modification of the protein. Isoelectric focusing of modified Hb S gave a series of bands identical in pattern to that observed for modified normal adult hemoglobin (Hb A).

To provide evidence that modification of hemoglobin by dibromoaspirin is due to covalent alteration by transacetylation, the reactions of the nonacetylated phenol, 3,5-dibromosalicylic acid, and of dibromoaspirin, at concentrations of 5 mM, with a 6% solution of cell-free Hb A were compared. Under these conditions, dibromoaspirin yielded essentially the same level of modification, 70–80%, as observed for the reaction with intracellular Hb A. On the other hand, with nonacetylated 3,5-dibromosalicylic acid, no modification was found.

Kinetic studies of the reaction between 5 mM dibromoaspirin and intracellular Hb A, as a 20% suspension of erythrocytes in isotonic phosphate buffer, revealed that 70–80% modification is achieved within 30 min. After that interval of time, no further increase in the extent of modification occurred and there was no change in the pattern of modified species separated by isoelectric focusing. Because this level of modification represents the utilization of less than 3 mM dibromoaspirin,* the remainder is presumably lost by hydrolysis. The spontaneous rate of hydrolysis of dibromoaspirin, $1.4 \times 10^{-4} \text{ min}^{-1}$ in isotonic phosphate buffer, pH 7.2, at 25° (J. Steele and I. M. Klotz, unpublished data) is far too slow to account for such a loss. Therefore, it seems probable that the hydrolysis is catalyzed by some constituent in erythrocytes, perhaps by hemoglobin. It has been shown previously (25) that aspirin is more readily hydrolyzed in the presence of erythrocytes than in plasma alone.

The reaction of dibromoaspirin was also studied with whole blood to determine the effects of competition by plasma proteins and of hydrolysis by esterases on the modification of intracellular hemoglobin. At a concentration of 5 mM, dibromoaspirin modified 20% of the hemoglobin in normal whole blood [hematocrit = 45% (vol/vol)]. In terms of the amount of

* The total concentration of Hb A in a 20% suspension of normal erythrocytes is approximately 1 mM. Assuming that bands 1 and 2 in Fig. 1 are due to derivatives doubly labeled per hemoglobin tetramer (22) and that the remaining bands anodal to unmodified Hb A correspond to more highly modified species, we calculate that less than 3 mM dibromoaspirin is consumed by transacylation of hemoglobin.

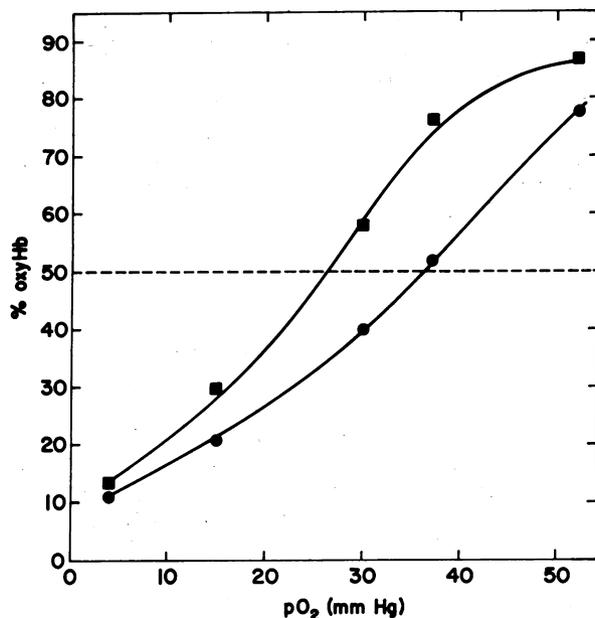


FIG. 2. Oxygen binding curves for sickle erythrocytes. Erythrocyte suspensions (20%, vol/vol) in isotonic phosphate buffer were either untreated (●—●) or exposed to 5 mM dibromoaspirin (■—■) for 2 hr at 37°. Erythrocytes were then washed and resuspended in human AB plasma prior to tonometer measurements as described (20).

hemoglobin modified, this value represents one-half to two-thirds the value observed in isotonic phosphate buffer.

Repeated dosage experiments were carried out to ascertain if the extent of modification with dibromoaspirin is cumulative. One dose at a very low concentration, 0.5 mM, of dibromoaspirin added to a 20% suspension of normal erythrocytes in isotonic phosphate buffer led to levels of modification just barely detectable by isoelectric focusing (<5%). However, with six such doses administered at hourly intervals, approximately 25% modification was achieved.

Modification by dibromoaspirin increased the oxygen affinity of both intracellular Hb A and Hb S. Modification of the hemoglobin with 5 mM dibromoaspirin in isotonic phosphate buffer produced a 25–30% decrease in the P_{50} (the partial pressure of oxygen at which 50% saturation of hemoglobin occurs) of both normal and sickle erythrocytes. Fig. 2 shows full-range oxygen-binding curves for untreated sickle erythrocytes and for sickle erythrocytes treated with 5 mM dibromoaspirin. After treatment with dibromoaspirin, the oxygen affinity of sickle erythrocytes, which is generally lower than that of normal erythrocytes (26), was brought into the range of normal cells.

Modification of sickle erythrocytes with dibromoaspirin produced a substantial decrease in erythrocyte sickling. Fig. 3 shows a plot of the percentage sickling versus oxygen saturation for untreated sickle cells and for sickle cells treated with 5 mM dibromoaspirin. At intermediate and low oxygen saturations, dibromoaspirin exerted a considerable antisickling effect. Photomicrographs of treated and untreated sickle erythrocytes (Fig. 4) clearly illustrate the decrease in erythrocyte sickling at low oxygen saturations. A decrease in the number of abnormally shaped cells other than the characteristic sickled form was also apparent. Similar results were found with sickled erythrocytes obtained from several different persons.

If the antisickling effect of dibromoaspirin is due to the acetylation of intracellular Hb S, then modified Hb S should manifest an increased MGC. Such an increase was indeed ob-

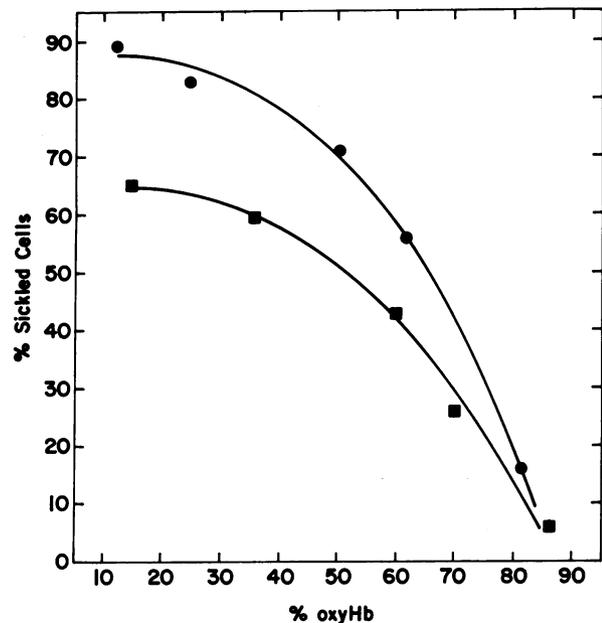


FIG. 3. The extent of erythrocyte sickling as a function of oxygen saturation. Suspensions (20%, vol/vol) of sickle erythrocytes were either untreated (●—●) or exposed to 5 mM dibromoaspirin (■—■).

served. The MGC of 60% modified Hb S was found to be 2–3 g/dl greater than that of unmodified Hb S (Table 1).

DISCUSSION

The reactivity of aspirin analogues in aminolysis by hemoglobin (Eq. 1) can be augmented in at least three ways: (i) by increasing the lability of the ester; (ii) by incorporating a functional group into the molecule to catalyze the aminolysis reaction; and (iii) by attaching groups to the molecule that will enhance binding of the drug to hemoglobin, preferably near the amino groups.

The present study with dibromoaspirin illustrates two of these features. The insertion of the electron-withdrawing Br atoms increases the reactivity toward small amines (e.g., propylamine) about 8-fold over that for unmodified aspirin (J. Steele, J. Walder, and I. M. Klotz, unpublished data). The observed augmentation in rate of acetylation of hemoglobin is much greater, however. Within 30 min, dibromoaspirin at 5 mM concentration modified 70–80% of Hb A or 60% of Hb S in erythrocytes (in 20% suspension). Under the same conditions, 20 mM aspirin produced less than 10% modification of Hb A. From this comparison, one can calculate that the overall rate of reaction of hemoglobin with dibromoaspirin must be at least 50 times that with aspirin. The additional increment in the overall rate, over that observed with propylamine, must reflect the enhanced binding of dibromoaspirin. The polarizable Br atoms would accentuate van der Waals and London interactions. It is also possible that some selectivity is generated from

Table 1. MGC of hemoglobins extracted from sickle erythrocytes

	MGC (g/dl)	
	No additions	With 5 mM dibromoaspirin
Patient A*	26.0	27.9
Patient B†	26.0	29.3

* 60% modified; 20% fetal hemoglobin.

† 60% modified; 15% fetal hemoglobin.

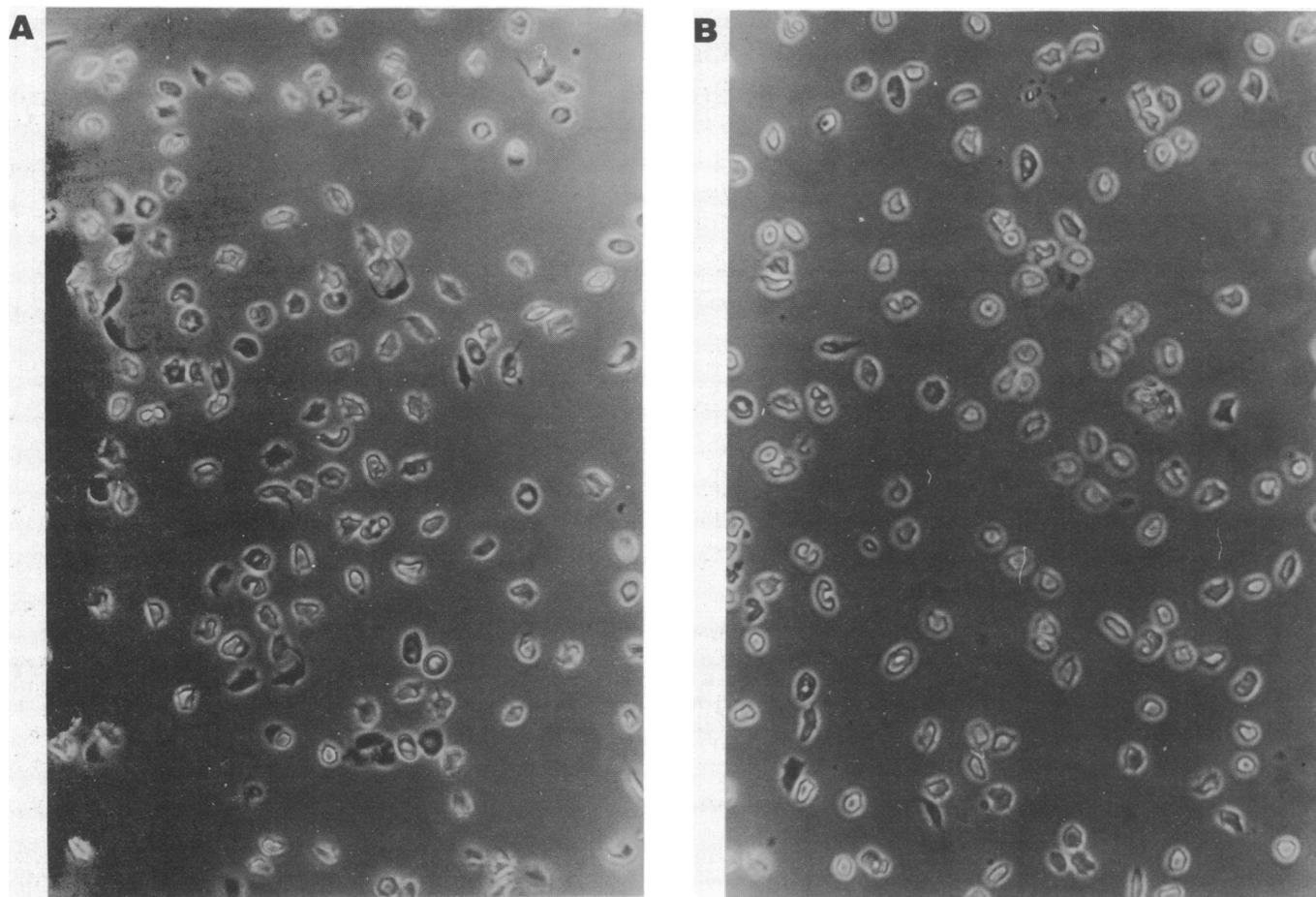


FIG. 4. Sickie erythrocytes after no treatment (*Left*) or after exposure to 5 mM dibromoaspirin (*Right*). Erythrocyte suspensions were equilibrated with a gas mixture containing 0.5% O₂, 5% CO₂, and the balance N₂ (pO₂ = 3 mm Hg) prior to fixation in isotonic 2% formalin. (×400.)

dipolar interactions of the aromatic C—Br bond with loci of cationic lysyl residues in the hemoglobin macromolecule.

The resolution of the dibromoaspirin-modified hemoglobin species by isoelectric focusing indicates that certain amino groups on the protein are modified preferentially. The three well-focused bands nearest unmodified Hb A (bands 1, 2, and 3 in Fig. 1B) account for approximately 80% of the modification. The sharpness of these bands suggests that each is due to a single hemoglobin species. Bands 1 and 2 probably contain protein that has been acetylated at a single site per dimer.† Band 3 may also correspond to a singly modified component. Alternatively, as suggested by the pattern of doublets observed in the densitometric scan of the gel, band 3 may be due to a doubly labeled derivative, acetylated at sites modified in both bands 1 and 2. In any event, acetylation by dibromoaspirin occurs predominantly at just 2 or 3 of the 23 distinct amino groups per hemoglobin dimer. This high degree of specificity is consistent with the notion that the enhanced activity of dibromoaspirin is due to binding by hemoglobin.

Modification of erythrocytes with dibromoaspirin increases the oxygen affinity of intracellular Hb A and Hb S to a quantitatively similar extent (Fig. 2). This increase in oxygen affinity in itself decreases erythrocyte sickling at intermediate oxygen

tensions because the fraction of Hb S in the deoxygenated form is decreased. Similar findings have been reported with cyanate (6, 12) and with various aromatic aldehydes (18, 20). These reagents, like dibromoaspirin, influence oxygen binding by modifying amino groups on the protein.

In addition to the effects on oxygen binding, treatment with dibromoaspirin directly inhibits erythrocyte sickling. That is, at equivalent oxygen saturation, the fraction of cells in the sickled conformation is decreased by treatment with dibromoaspirin. This is illustrated in plots (Fig. 3) of the percentage sickling versus oxygen saturation for untreated cells and for cells treated with 5 mM dibromoaspirin. The photomicrographs in Fig. 4, of treated and untreated erythrocytes at low oxygen saturation, reveal further the marked inhibition of erythrocyte sickling with dibromoaspirin. That this effect is due to acetylation of Hb S is supported by the observation that the MGC for deoxy Hb S extracted from treated erythrocytes is significantly increased (Table 1).

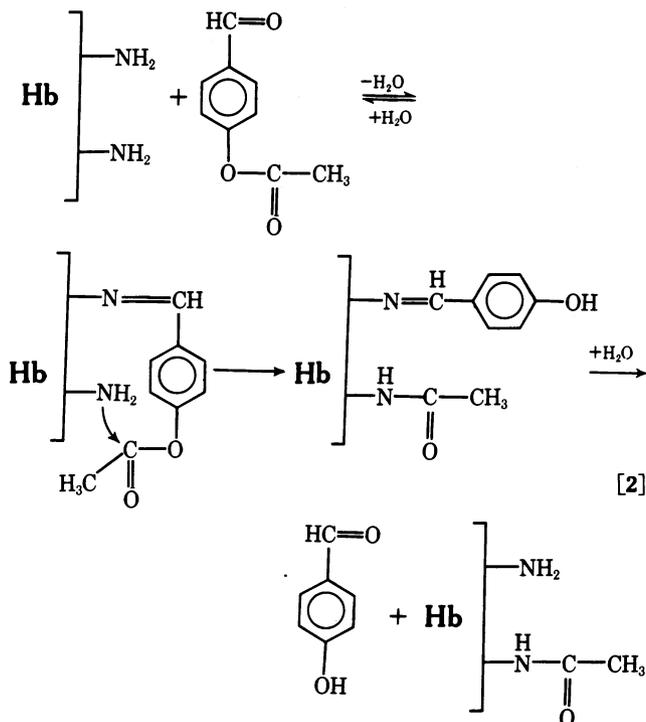
In all of these studies, approximately 60% of the Hb S had been modified by dibromoaspirin. At higher levels of modification, as might be achieved by repeated dosage regimens *in vivo*, correspondingly greater effects on both sickling and gelling would be anticipated. Our studies of the reaction of intracellular hemoglobin with dibromoaspirin performed in whole blood and those done with the repeated dosage regimen strongly suggest that high levels of modification of Hb S by dibromoaspirin can be achieved also *in vivo*. Because of the obvious structural similarity of dibromoaspirin to aspirin, we

† As Bridges *et al.* (22) have pointed out, asymmetrically modified hemoglobins having one acetyl group per tetramer would not be isolable by isoelectric focusing because the protein would dissociate into unlike dimers that would sort with other dimers of like charge.

do not expect the toxicity of dibromoaspirin to be proscriptive.†

In contemplating directions for further improvements in antisickling compounds, we can take cognizance of the features introduced in dibromoaspirin. If the augmentation in its effectiveness is due primarily to its increased polarizability interactions, then the corresponding iodo derivative may be even more efficacious. On the other hand, if electron-withdrawing ability is the dominant factor, a chloro or fluoro derivative should be more potent than the bromo compound. We recognize also that an extremely labile acylating agent may even fail to transacetylate amino groups of hemoglobin because the reagent may be depleted very rapidly by hydrolysis. This behavior is illustrated by 2-acetoxybenzaldehyde: the hydrated aldehyde in the *ortho* position accelerates enormously the hydrolysis of the acetoxy moiety (27, 28).

Another approach that seems particularly promising, in the light of work on the binding of aldehydes to hemoglobin by Schiff base linkages (7, 15, 20), is to combine within a single reagent the potential for Schiff base formation with that for transacylation. For example, *p*-acetoxybenzaldehyde should have a labile acetoxy moiety and an aldehyde group capable of forming a Schiff base adduct:



The Schiff base linkage should anchor the reagent on the hemoglobin surface and, by increasing the local concentration of acylating reagent, substantially augment its effectiveness in reacting with an amino group of hemoglobin. Clusters of amino groups would favor reactions of this type and hence introduce an element of specificity in transacetylation of hemoglobin.

It is thus apparent that rational variations in the structure of

† The requirement for biological acceptability led us initially to investigate aspirin itself. Similarly, we have also examined several esters of *p*-acetamidophenol (Tylenol).

2-acetoxybenzoic acid, aspirin, open access to a range of molecules that could be potent antisickling reagents.

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