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Inhibition of Erythrocyte Sickling by Thiol Reagents

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SUMMARY

The antisickling effects of eight thiol reagents that cross the red cell membrane and then react with the cysteine β93, the only accessible thiol group of hemoglobin, have been investigated at various pO_2 values. In spite of completely reacted hemoglobins, the potent antisickling effect varied from one compound to the other and was partially related to the extent of the increased oxygen affinity of intact sickle cells induced by these compounds. The formation of methemoglobin upon the incubation of red blood cells with some disulfides had only a small effect on the sickling process.

INTRODUCTION

In sickle cell disease, HbS[1] aggregates upon deoxygenation to form helical fibers which distort the cell into a variety of unusual shapes and increase blood viscosity. These sickled erythrocytes are less deformable than normal red blood cells and, as a result, tend to occlude the microcirculation, leading to tissue injury and necrosis.

One of the therapeutic approaches to reverse the sickling process involves modifying the behavior of sickle hemoglobin (HbS) with reagents that cross the red cell membrane and bind to intracellular HbS in a manner that diminishes fiber formation. The inhibition of sickling can be achieved either by preventing fiber formation directly, by increasing the oxygen affinity of HbS, or by decreasing the hemoglobin concentration. The artificially increased oxygen affinity of HbS reduces the deoxy-HbS concentration and, as a consequence, decreases the proportion of HbS polymerized at a given pO_2.

Many reagents that react covalently with hemoglobin and increase its oxygen affinity have been studied for their ability to inhibit sickling. At present such compounds fall into three major categories: those that carbamoylate or acylate (1–3), those that form Schiff base adducts (4–6), and those that modify sulfhydryl groups, of the hemoglobin tetramer (7, 8). The targeting of cysteine β93 with thiol reagents has been investigated in our laboratory because of the location of this residue in a region involved in the conformation and the functional modulation of the hemoglobin molecule. Thiol reagents are bound under most conditions exclusively to the cysteine β93 of Hb. The effect of many thiol reagents is an increase in the oxygen affinity of hemoglobin, a decrease in the alkaline Bohr effect, and for some of them, a reduction in heme-heme interactions (9–13). The modifications of functional properties of hemoglobin are highly dependent on the structure of the compound bound to the cysteine β93 residue. The reactivity of the thiol reagent toward Hb is also highly dependent on the charge and the structure of the compound and of the amino acid residues surrounding the cysteines β93 (13).

Previous results from our laboratory have demonstrated that the disulfide cystamine, which reacts with intracellular HbS, is an antisickling agent. The antisickling effect is related mostly to an increase in the oxygen affinity of HbS (7).

We report here the antisickling effects of eight thiol reagents that cross the red cell membrane, taking into consideration the proportion of reacted Hb, the extent of the modifications of the functional properties of Hb and the extent of methemoglobin formation.

EXPERIMENTAL PROCEDURES

Materials. Cystamine dihydrochloride, NEM, PHMB, and IAA were purchased from Sigma. L-CDE, 2,2′-dithiodibenzoic acid, DTNB, 2-DTP, and tetraethylthiuram disulfide (disulfiram) were obtained from Fluka. 4-APD was obtained from Aldrich, 1,1′-dithiodiformamidine was from Serva, 4-DTD was from Eastman, and DMSO was from Merck.

Carrier ampholytes (ampholines LKB, pH 6 to 8 and pH 7 to 9) for isoelectric focusing were obtained from LKB, acrylamide and bisacrylamide were from Fluka, ammonium persulfate was from Merck, and *N,N,N′,N′*-tetramethylethylenediamine was from Sigma. All chemicals were at least analytical grade.

Chemical modifications of intracellular hemoglobin. Whole blood was drawn by venipuncture from normal adults and homozygotes for sickle

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[1] The abbreviations used are: HbS, hemoglobin S; DMSO, dimethyl sulfoxide; NEM, *N*-ethylmaleimide; IAA, iodoacetamide; L-CDE, L-cystine dimethyl ester; DTNB, 5,5′-dithiobis(2-nitro)benzoic acid; 2-DTP, 2,2′-dithiodipyridine; tetraethylthiuram disulfide (disulfiram); 4-APD, 4-aminophenyl disulfide; 1,1′-dithiodiformamidine; 4-DTD, 4,4′-dithiodimorpholine; MCHC, mean corpuscular hemoglobin concentration; PHMB, *p*-hydroxymercuribenzoate; MGC, minimum gelling concentration.

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cell anemia. EDTA was used as anticoagulant (7) and was tested to maintain a constant level of 2,3-diphosphoglycerate in the erythrocyte during a minimum period of 7 days at 4°. Erythrocytes were washed three times in phosphate buffer (0.15 M sodium phosphate, pH 7.4). Packed erythrocytes were suspended to a final hematocrit of 5% (v/v) in phosphate buffer. All compounds were tested at a concentration of 5 mM. After addition of the reagent, samples were incubated 1 hr at 37°. When used, the final concentration of DMSO was 0.28 M. At the end of the reaction, the suspension was centrifuged, and the cells were washed three times at 4° in phosphate buffer to eliminate excess reagent.

In most cases, the extent of modification of hemoglobin could be assessed by isoelectric focusing on slabs of acrylamide gels performed as described previously (14). Modified hemoglobins appeared as focused bands distinct from those of unmodified hemoglobin. The percentage of the reacted fraction was determined by densitometry with a Cello system (Sebia, Issy-les-Moulineaux, France) as previously described (15).

In some cases, the modified and unmodified fraction had identical pI values and could not be separated by isoelectric focusing. In such circumstance, the percentage of reacted hemoglobin was determined by the titration of the free sulfhydryl groups of Hb according to the method of Boyer (16). Before titration, the hemoglobin solutions were passed through a Sephadex G-25 column to remove excess reagent and derivatives which could interfere with the assay of hemoglobin thiol groups.

Oxygen dissociation measurements. The oxygen dissociation curves of normal red blood cells were recorded on the Aminco Hem-O-Scan at 37°. Treated erythrocytes were washed three times with sodium phosphate buffer, pH 7.4, and resuspended in the same buffer to a final hematocrit of 50% (v/v). The percentage of methemoglobin was determined at the end of each experiment according to Benesch *et al.* (17) with a Cary 118C spectrophotometer. When sickle blood was studied, all the oxygen dissociation curves were performed on the same day, with the same blood sample for all reagents. This experiment was done on three different occasions with different patients.

In vitro sickling experiments. The blood samples used for the oxygen dissociation curves also were used for the sickling experiments. The results were similar on three occasions. One set of results is presented. At the end of each incubation with a thiol reagent, red cells were washed three times, and packed erythrocytes were suspended to a final hematocrit of 5% (v/v) in sodium phosphate buffer, pH 7.4. An aliquot of 500 μl of the cell suspension was placed in a 10-ml flask and equilibrated with humidified gas mixtures at various partial pressures of oxygen. Mixtures were obtained using a proportional pump (Wösthoff, Bran et Lubbe, France). The samples were equilibrated at a given pO_2 for 20 min at 37° in a rotary shaker, and the cells were then fixed by transfer of the suspension into a solution of 3% formaldehyde in phosphate buffer equilibrated at 37° and at a partial pressure identical to that of the red cell suspension. The red cell morphology was examined with an Olympus microscope equipped with differential interference contrast optics. A minimum of 500 fixed cells was counted for each sample. The percentage of deformed cells present in oxygenated sample was not subtracted; the accuracy of measurement was 5% (18).

Methemoglobin was induced in red blood cells by incubating whole blood with sodium nitrite at a molar ratio Hb heme/nitrite = 4 for 5 min at 37°. At the end of the incubation, the red cells were washed three times in phosphate buffer in order to eliminate excess nitrite. The degree of sickling and oxygen affinity (P_{50}) were measured immediately thereafter according to the methods described above (17, 18).

Polymerization studies. HbS used for this study was purified by DEAE-cellulose chromatography (19). HbS (0.2 mM) was subsequently reacted in 0.1 M sodium phosphate buffer, pH 7.4, with NEM and IAA with a molar ratio, compound/Hb, of 10 and 20 during 30 min at 37° in a rotary shaker. At the end of the incubation, the mixture was concentrated under vacuum and dialyzed against 0.15 M potassium phosphate buffer, pH 7.35, in order to eliminate the excess of the thiol reagent. The complete modification of hemoglobin by the reagents was checked by isoelectric focusing. Solubility (C_{sat}) measurements were performed by the ultracentrifuge assay under the conditions used by Benesch *et al.* (20).

RESULTS

For the sake of clarity, we artificially divided the compounds into two groups according to the presence or the absence of methemoglobin formation.

Group 1: inhibition of sickling without metHb formation. The reaction of 5 mM thiol reagents with 5% suspension of normal erythrocytes in phosphate buffer, pH 7.4, at 37° for 1 hr led to a variable percentage of modification of intracellular Hb depending upon the compound tested.

Table 1 shows the results obtained with five reagents which induced 95–100% modification of intracellular Hb in both normal and sickle cells. The isoelectric focusing pattern of modified HbS was similar to that observed for modified normal HbA shown previously (13).

The thiol reagents increased to various extents the oxygen affinity of both intracellular HbA and HbS. Complete modification of intracellular HbS with iodoacetamide, cystamine, and 2,2′-dithiodipyridine increased the oxygen affinity of sickle cells, their P_{50} becoming close to that of untreated, normal erythrocytes. The increase in oxygen affinity of the intact red blood cells was most pronounced for NEM (73%) and 4,4′-dithiodimorpholine (76%) in sickle cells.

These thiol reagents produced different and substantial decreases in erythrocyte sickling at various pO_2 values, as shown in Fig. 1, but not in the absence of oxygen. The strongest inhibition of sickling was observed with NEM and 4,4′-dithiodimorpholine. In contrast, the antisickling effect obtained with cystamine was much smaller in spite of the >95% modification of intracellular Hb, a proportion similar to that induced by the other thiol reagents.

These results suggest that the inhibition of sickling induced by these thiol reagents was mainly due to the increased oxygen affinity of HbS, because the antisickling effect vanished at $pO_2 = 0$. However, in the experimental conditions used, a moderate effect on the polymerization process by contact inhibition of the HbS

TABLE 1

Modifications of the functional properties of normal and sickle cells by thiol reagents: (percentage modification of cellular Hb was 95% or greater)

Compound	P_{50} of red blood cells		Change in P_{50}	
	Normal cells	Sickle cells	Normal cells	Sickle cells
	mm Hg		*%*	
Control	28[a]	55[a]		
NEM	8	14.5	71	73
2,2′-Dithiodipyridine	14.5	32	48	42
Idodoacetamide	14	24.5	50	55
4,4′-Dithiodimorpholine	10	13	64	76
Cystamine	15.5	32.5	45	41

[a] Each value is the mean of triplicate experiments; the standard error of the mean is less than 5% of the mean value in all cases.

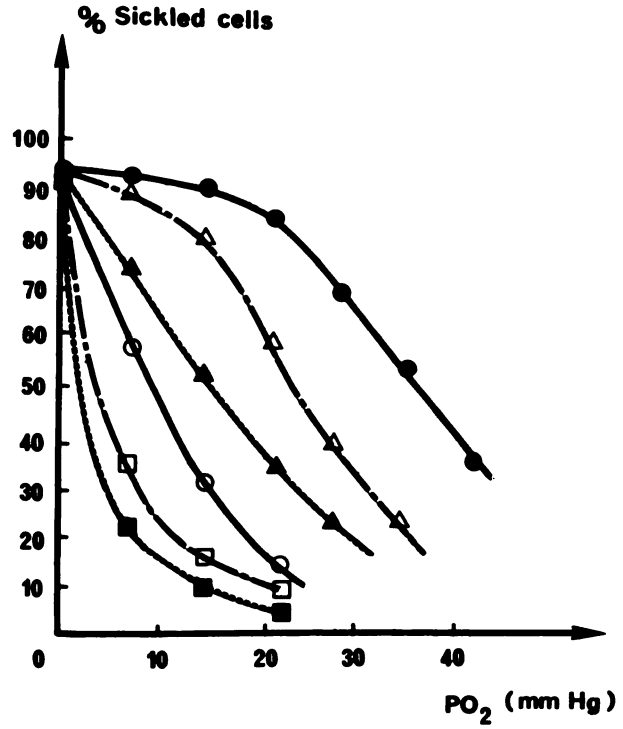

FIG. 1. *Sickling curves of sickle cells (●——●) and sickle cells treated with 5 mM cystamine (Δ-·-Δ), iodoacetamide (▲---▲), 2,2′-dithiodipyridine (○——○), 4,4′-dithiodimorpholine (□-·-□), N-ethylmaleimide (■--■)*

molecule cannot be ruled out at $pO_2 = 0$. Both mechanisms shift the oxygen dissociation curve of sickle cells to the left.

To determine the role played by the increase in the oxygen affinity in the antisickling effects of thiol reagents, normal cells were incubated with thiol reagents under identical conditions, and the oxygen dissociation curve was determined. As shown in Fig. 2, the inhibition of sickling of sickle cells at 21 mm Hg pO_2 was correlated to the P_{50} of normal cells ($r = 0.93$; $p < 0.0005$), which do not sickle and do not contain polymerized hemoglobin, indicating that the sickling inhibition can be attributed to the increased oxygen affinity of sickle cells. Similar results were obtained with sickle cells ($r = 0.90$; $p < 0.0005$). However, three compounds, 4-DTD, 4-APD and 2-DTP, which induced similar inhibition of sickling had very different effects on the P_{50} of sickle cells, ranging from 11 to 33 mm Hg. In contrast, the P_{50} values of normal cells induced by the same reagents were very similar. In addition, sickle cells incubated with 2-DTP exhibited a P_{50} similar to that of cells treated with cystamine, but the inhibition of sickling was much greater with 2-DTP-treated cells. Accordingly, it can be deduced from this experiment that 2-DTP inhibits sickling by another mechanism in addition to the increase of the oxygen affinity of HbS. Another means to detect antisickling contribution by contact inhibition of the HbS molecule is to study the antisickling effects of thiol reagents at a given oxygen saturation of hemoglobin. The results that support this direct effect of same thiol reagents will be shown below.

Group 2: inhibition of sickling by thiol reagents that oxidize Hb. Three thiol reagents, 4-aminophenyl disulfide, disulfiram, and L-cystine dimethyl ester, induced methemoglobin formation in red blood cells. Previous results have shown that the oxidation of the heme group is not related to the binding of these reagents to the cysteine β93 residue, because the oxidation of hemoglobin is similar to that of Hb previously reacted with NEM (13). The amounts of methemoglobin induced by these reagents differ markedly from one compound to the other, with the highest proportion of methemoglobin obtained with 4-aminophenyl disulfide in both normal and sickle erythrocytes. Table 2 summarizes the effects of these three thiol reagents on the oxygen affinity of Hb and on methemoglobin formation. The presence of 27% methemoglobin induced by nitrite in normal red blood cells decreased the P_{50} value only slightly from 28 to 24 mm Hg. Similarly, the presence of 27% metHb in sickle

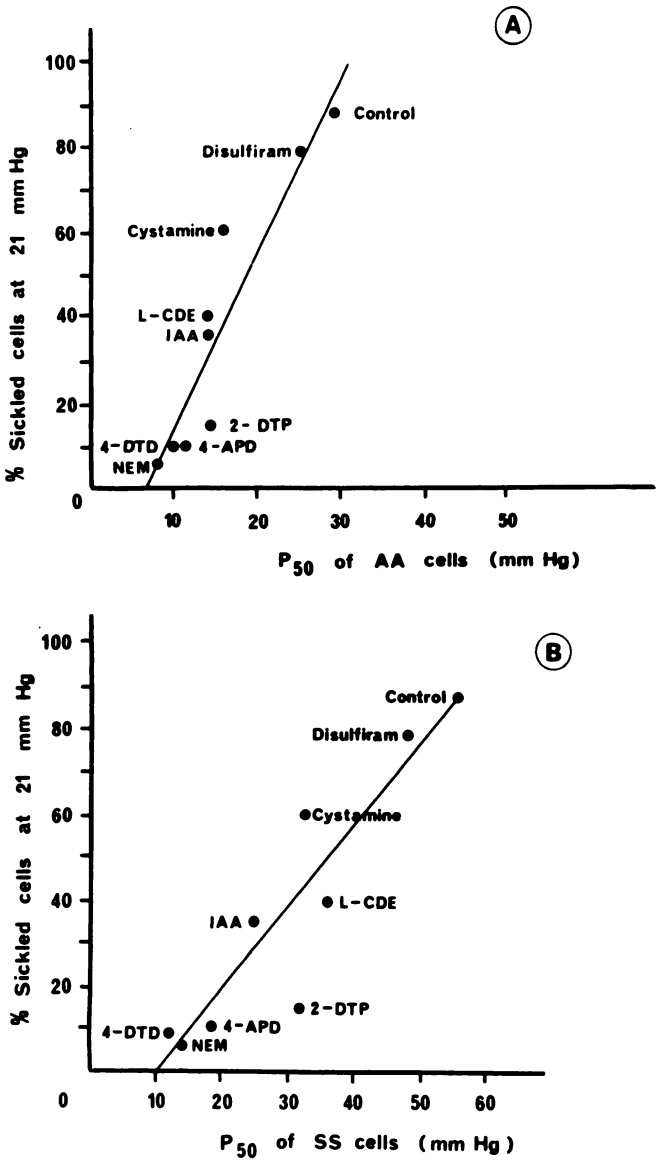

FIG. 2. *Comparison between the percentage of sickled and deformed cells of sickle cells at 21 mm Hg and the P_{50} of normal cells (A) and sickle cells (B) upon the addition of thiol reagents.*

TABLE 2

Modification of the functional properties of normal and sickle cells by thiol reagents which induce oxidation of intraerythrocytic Hb

Compound	Modification of Intraerythrocytic Hb		P_{50} of red blood cells		MetHb		Change in P_{50}	
	HbA	HbS	Normal cells	Sickle cells	Normal cells	Sickle cells	Normal cells	Sickle cells
	%		*mm Hg*		%		%	
Control			28[a]	55[a]	0	0		
Control (27% metHb)			24	50	27	27	8.6	9
4-Aminophenyl disulfide (3 mM)	80	90	11	18	30	29	61	67
Disulfiram[b]	80	90	25	49	16	17	11	11
L-Cystine dimethyl ester	75	90	14	36	19	15	50	35

[a] Each value is the mean of triplicate experiments; the standard error of the mean is less than 5% of the mean value in all cases.
[b] Compound dissolved in DMSO solution (0.28 M).

cells decreased the P_{50} from 55 to 50 mm Hg. In contrast, 4-aminophenyl disulfide, which gave rise to similar levels of methemoglobin, dramatically increased the oxygen affinity of normal cells and of sickle cells. L-Cystine dimethyl ester, which exhibited a lower potential for methemoglobin formation, also induced a major shift of the oxygen dissociation curve of normal and sickle cells. By contrast, disulfiram had a negligible effect on the P_{50} of intact cells despite significant methemoglobin formation. Thus, various modifications of the functional properties of normal cells and sickle cells induced by different thiol compounds were related mainly to their covalent binding to hemoglobin and to a much lesser extent to the level of metHb formation.

Figure 3 shows the inhibition of sickling induced by the three compounds that induced methemoglobin formation within the cells. In control experiments, 27% metHb inhibited the sickling process only slightly. Accordingly, the slight inhibition of sickling obtained with disulfiram, which did not modify the P_{50} of Hb, could be related to the presence of 17% metHb. In contrast, 4-aminophenyl disulfide inhibited sickling to an extent much greater than could be accounted for by the 29% metHb. The inhibition of sickling obtained with these three compounds also correlates with the modification of the oxygen affinity of sickle cells, as shown in Fig. 2.

The heterogeneity of the effects of thiol reagents on hemoglobin function extended to observations on the membranes of red blood cells. Some compounds such as DTNB or PHMB, which react with Hb in solution, are not bound to intracellular Hb. This result indicates that these compounds do not cross the red cell membrane. Certain other compounds such as 1,1′-dithiodiformamidine and 2,2′-dithiodibenzoic acid, which modify the shape of normal and sickle cells, produce echinocytes. Consequently, their inhibition of sickling cannot be evaluated under the experimental conditions employed. In addition, compounds that induced hemolysis, such as PHMB or 4,4′-dithiodipyridine, were not studied.

Inhibition of sickling related to oxygen saturation. Figure 4 shows the relationship between the percentage of sickling and the oxygen saturation for untreated sickle cells and for sickle cells treated with 5 mM of various thiol reagents. All compounds except cystamine and disulfiram exhibited an antisickling effect additional to that induced by the change in oxygen affinity of sickle cells. The maximum direct antisickling effect was induced by NEM.

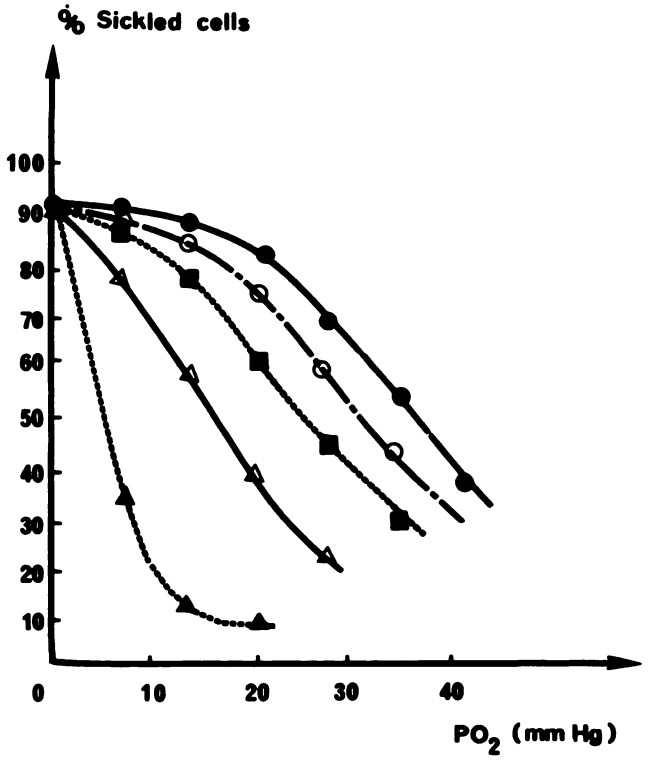

FIG. 3. *Sickling curves for sickle cells treated with thiol reagents which induce methemoglobin formation*
●——●, untreated cells; ■- - -■, control + 27% metHb; O-·-O, disulfiram (metHb: 17%); Δ——Δ, L-cystine dimethyl ester (metHb: 15%); ▲- - -▲, 4-aminophenyl disulfide (metHb: 29%).

Polymerization studies. In such studies, complete deoxygenation of samples with high Hb concentration was obtained with sodium dithionite (56 mM). This compound reduced mixed disulfides of Hb. Consequently, only NEM and IAA could be used to investigate their inhibitory effect on polymerization of deoxy-HbS.

The increased solubility of deoxy-HbS modified by these two thiol reagents is compared with HbS in Table 3. The greater increase in solubility of HbS was obtained

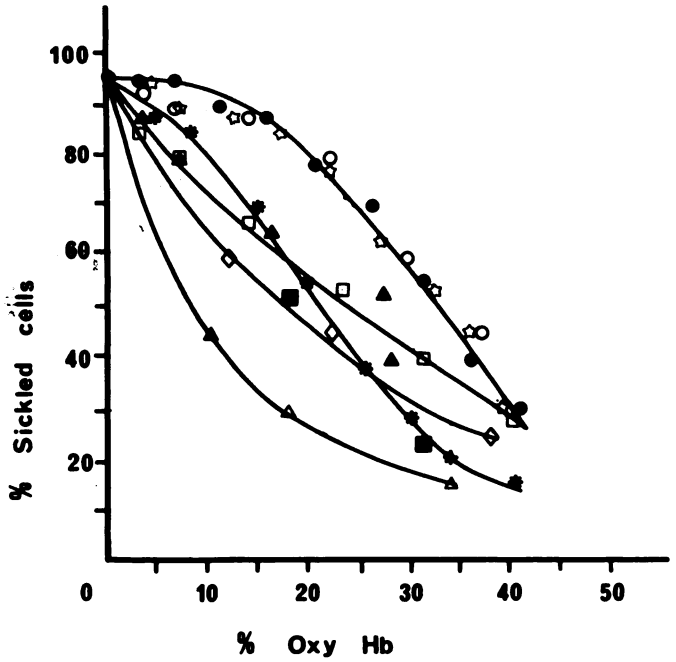

FIG. 4. *Influence of oxygen saturation of sickle red blood cells on sickling of control (●) and sickle erythrocytes treated with 5 mM (■) 4-aminophenyldisulfide, (☆) disulfiram, (△) NEM, (▲) iodoacetamide, (□) 2,2′-dithiodipyridine, (◇) 4,4′-dithiodimorpholine, (*) L-cystine dimethyl ester, and (○) cystamine*

TABLE 3

The influence of thiol reagents on the solubility of HbS gels

Measurements were carried out at 30° and pH 6.8 in the presence of 56 mM sodium dithionite.

Hemoglobin	Hemoglobin concentration		
	Initial	Supernatant	Pellet
		g/dl	
HbS	26.4	16.0	48.7
	26.5	15.8	49.5
HbS modified by NEM	25.7	21.2	50.4
	26.2	21.5	49.8
HbS modified by IAA	26.5	17.3	49.0
	26.6	19.2	49.0

with NEM. Nevertheless, no modification of the pellet concentrations compared to HbS could be observed, either with NEM or IAA, suggesting that the polymer structure is not greatly modified.

DISCUSSION

The present study provides evidence that thiol reagents are, to various extents, inhibitors of sickling. Under the conditions used, the mechanism underlying the antisickling effect is complex and involves changes in the oxygen affinity of hemoglobin, inhibition of contact formation in the hemoglobin S polymer, and possibly other modifications of the red blood cell. The greatest inhibition of sickling was obtained with the compounds that induced the largest increase in oxygen affinity. A good correlation was obtained between the increase in the oxygen affinity and the inhibition of sickling ($p < 0.0005$).

The mechanism underlying this increased oxygen affinity is the destabilization of the T conformation and the shift of the R ↔ T equilibrium toward the R state. As previously shown for *N*-substituted maleimides, closely related reagents yield hemoglobin derivatives having very different functional properties (21, 22) and structure (23, 24). These differences implicate specific interactions of the reagents bound to the cysteine F9(93)β with adjacent amino acid side chain residues. Several amino acid residues surrounding the thiol group are involved in the oxygen affinity, in the alkaline Bohr effect and in the cooperativity (25).

The bifunctional reagent bis(*N*-maleimidomethyl) ether exhibited a potent antisickling effect (8) and locked the hemoglobin structure in the R state (26). This compound is bound covalently to His FG4(97)β in addition to cysteine F9(93)β.

The functional properties of the hemoglobin derivatives confirm the great diversity of the effects of thiol reagents covalently bound to a single target and will be published separately.

The inhibition of sickling by destabilization of the T state or deoxy quaternary structure of HbS also can be obtained with other covalently bound reagents. These mainly involve compounds that bind to the α amino terminal valine in either the α or the β chain. Reagents such as cyanate, methylisocyanate, and carbamyl phosphate are carbamoylating agents which react predominantly with the N terminus of the α chains. Pyridoxylation by reagents such as 5′-pyridoxal sulfate and 5′-deoxypyridoxal occurs exclusively at the N terminus of the α chains, while that for 5′-pyridoxal phosphate occurs at the N terminus of the β chains (4, 27). Modification by pyridoxal reagents which react with the N terminus of the α chains results in increased O_2 affinity and inhibition of sickling.

In addition to the destabilization of the T structure, the inhibition of sickling by some thiol reagents may depend upon other mechanisms such as direct inhibition of the formation of intermolecular contacts in the fibers, diminution of the intracellular Hb concentration, or modification of the erythrocyte membrane. While cysteine β93 does not participate directly in the contacts reported by Wishner and Love (28, 29), residues serine β89 and glutamate β90, which have been implicated in such contacts, are located near cysteine β93.

Furthermore the two residues Phe β85 and Leu β88, which are implicated in the receptor site of Val β6, are located in the same region between E and F helices as cysteine β93. Previous results obtained with cystamine have shown a slight increase in the minimum gelling concentration of reacted HbS (MGC = 26.6 g/dl) compared to untreated HbS (MGC = 23.9 g/dl) (7) which suggested an inhibition of polymerization by modification of residue cysteine β93. Recently, this study has been repeated by Chang *et al.* (30), who did not observe any inhibition of polymerization of HbS by cystamine in the absence of oxygen. However, they used dithionite to complete the deoxygenation and thereby reduced the

disulfide bridge of the cystamine-reacted hemoglobin. Consequently, one would not expect any increase of the solubility of treated hemoglobin S in such conditions.

Further evidence for the inhibition of sickling, by an oxygen affinity-independent mechanism, is provided by Fig. 4. The percentages of sickled cells induced by the thiol reagents vary widely at the same degree of oxygen saturation of HbS. These differences suggest that at least two processes are involved and that disulfiram and cystamine act mainly by increasing the oxygen affinity of HbS, while other reagents act by both an oxygen affinity-mediated mechanism and by one involving inhibition of intermolecular contacts.

A direct evidence of the inhibition of HbS polymerization is provided by the increased solubility of HbS (C_{sat}) reacted with thiol reagents (IAA and NEM). In addition the results show that the hemoglobin concentration of the pellets formed from HbS modified by the two thiol reagents are identical to that from control HbS. Therefore, it appears that the structure of the fiber was not modified by these thiol reagents.

In an earlier study performed with cystamine (7), a slight increase in the mean corpuscular volume with a concomitant decrease in MCHC was observed. Such dilution of the intracellular Hb also can inhibit sickling. Most of the thiol reagents tested react with membrane SH groups and consequently modify the membrane permeability and transport systems of the red cells. In this regard, it is noteworthy that the thiol reagents DTNB and PHMB did not react with intracellular Hb and, consequently, had no effect either on sickling or on the oxygen affinity of sickle red cells (see ref. 13).

Recently, Sato and Ohnishi (31) showed that the induction of stomatocytes and the antisickling effect of chlorpromazine was inhibited by *N*-ethylmaleimide and by iodoacetamide. Any antisickling activity of these compounds at the membrane level would have enhanced rather than inhibited the antisickling effect of chlorpromazine.

As shown in Table 2, several compounds induced methemoglobin formation in both normal and sickle cells. Identical percentages of metHb were observed in the two types of cells. It has long been recognized that the methemoglobin (or cyanmethemoglobin) form of HbS will not readily undergo gelation and sickling because the T conformation of the hemoglobin is necessary for polymerization. Studies by Beutler (32) have demonstrated that methemoglobin induced *in vivo* by the administration of sodium nitrite or *para*-aminopropriophenone inhibits the sickling process only slightly. The inhibition of sickling obtained in our experimental conditions with 27% metHb is similar to that obtained by Beutler's group.

As shown recently, metHbS inhibits polymerization of deoxy-HbS in an intermediate manner between fully liganded (Co-Hb) and deoxy-HbS. A solution of 100% metHbS polymerizes at high concentration (36 g/dl) (33). Since metHbS has a conformational equilibrium intermediate between oxy- (or carbonmonoxy-) and deoxy-HbS (34), its participation in the deoxy-HbS polymer is related to the approximation of the HbS derivative toward the deoxy conformation. In addition, the slight effect of metHb on the inhibition of sickling can be explained by the presence in the red blood cells of symmetrical hybrids $\alpha_2^{met}\ \beta_2^S$ and $\alpha_2\beta_2^{S-met}$. Such hybrids can be compared to the half-liganded hybrid tetramer described by Bookchin and Nagel (35, 36). The deoxy conformations of these molecules resemble, but are in a state not identical to, the T state. Such hybrids have been detected by isoelectric focusing (results not shown) (13). In addition, Table 2 shows that 27% metHb exerts little effect on the oxygen dissociation curve of normal and sickle erythrocytes. This result is in agreement with the findings of Darling and Roughton (37) for normal red blood cells and those obtained by Beutler (32) for sickle cells. The functional properties of symmetrical hybrid having one chain in the ferric form and one chain in the ferrous form have been studied by Banerjee and Cassoly (38). The results show that the oxygen affinity is enhanced and the cooperativity is abolished with $n = 1$. The slight increase in the oxygen affinity of red blood cells in the presence of 27% metHb can be the consequence of the presence in the cells of such molecules. Consequently, metHbS and symmetrical hybrids inhibit polymerization mainly by dilution of deoxy-HbS molecules. Inasmuch as the two compounds 4-aminophenyl disulfide and L-cystine dimethyl ester profoundly increased the oxygen affinity of both normal and sickle erythrocytes but produced a level of metHb no greater than that for the control (27% metHb), it can be concluded that the inhibition of sickling observed with these two compounds may be attributed principally to the binding of each thiol reagent to cysteine β93 and only marginally to the presence of metHb. Only the minor effects obtained with disulfiram can be related solely to the presence of metHb because this reagent exhibited little effect on the oxygen affinity of normal cells.

As shown previously (13), metHb is not induced by the binding of the thiol reagent to the cysteine β93 residue *per se* because a similar degree of metHb formation occurs when Hb is alkylated at this position.

In conclusion, the linkage of a thiol reagent to cysteine β93 induces a great variation of the antisickling effect depending on the reagent bound to HbS. Despite the widely distributed thiol groups on proteins with a greater reactivity than hemoglobin, the present experiments show that the vicinity of cysteine β93 can be used to improve the antisickling effect of thiol reagents. This rationale is now being used to design potent antisickling agents with greater specificity for hemoglobin and to bind intracellular glutathione to HbS through an intermediate reaction. Hemoglobin-glutathione mixed disulfide has been found to be a very potent antisickling agent (39).

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