Inhibition of erythrocyte sickling by cystamine, a thiol reagent
(hemoglobin S polymerization/oxygen affinity/intracellular hemoglobin concentration)

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ABSTRACT Incubation of sickle cells with cystamine, a thiol reagent, resulted in the formation of an intracellular S-ethylamine derivative. The rate of the reaction was dependent upon the cystamine concentration, the temperature, and the duration of the incubation. The cystamine-treated cells demonstrated a marked inhibition of sickling under hypoxic conditions, a decrease in their mean corpuscular hemoglobin concentration, and a significant increase in their oxygen affinity. The oxygen affinity of these cells was less dependent on their mean corpuscular hemoglobin concentration than that of untreated sickle cells. The minimum gelling concentration of S-ethylamine deoxyhemoglobin S was slightly increased. Cystamine did not affect the intracellular pH or the 2,3-diphosphoglycerate level. The exact contribution of the interrelated factors in cystamine inhibition of sickling (changes in oxygen affinity, mean corpuscular hemoglobin concentration, and minimum gelling concentration) has yet to be determined.

Under hypoxic conditions, hemoglobin S (Hb S) molecules polymerize either in concentrated solutions (1, 2) or within erythrocytes (3, 4), giving rise to sickled cells. The polymerization of Hb S within the erythrocytes results in an abnormally low oxygen affinity (5, 6), which is dependent on Hb concentration within the cells containing Hb S (cells) (7).

Several agents have been reported to inhibit sickling, such as cyanate (8), nitrogen mustard (9), alkylurea (10), dimethyl adipimide (11), and, more recently, bis(N-maleimidomethyl) ether (12). Cystamine, a thiol reagent (NH₂CH₂CH₂CH₂S-SCH₂CH₂-NH₂), has been shown to bind to Hb (13). The S-ethylamine Hb derivative migrated as a single electrophoretic band distinct from that of the unreacted Hb. This property provided a simple means of quantitation of the cystamine-reacted fraction. The S-ethylamine Hb exhibited a high oxygen affinity and a reduced Bohr effect (14). In view of these properties, it was reasonable to suspect that cystamine could exert an antischickling effect.

In the present report, the effects of cystamine on the sickling of S cells and on some related phenomena are presented.

MATERIALS

Fresh blood samples from individuals homozygous (SS), heterozygous (AS), and doubly heterozygous (SC) for sickle hemoglobinopathy and from normal adults were drawn into EDTA as anticoagulant. The composition of this anticoagulant was 1% EDTA, 0.6% NaCl, and 6% glucose (wt/vol); it maintained a constant level of 2,3-diphosphoglycerate (P₂G) in the erythrocytes during a minimum period of 7 days at 4°. Cystamine dichloride (2,2-dithio-bisethylamine dichloride) was provided by Merck, purified glutaraldehyde (25% aqueous solution) was obtained from TAA Laboratories, and enzymes and substrates were obtained from Boehringer. Cellulose acetate was supplied by Chemetron (Milano), and Sephadex by Pharmacia. All chemicals were of analytical grade.

METHODS

Cystamine dichloride (0.22 M) was dissolved in 0.15 M sodium phosphate solution. The solution was adjusted to pH 7.4 with monosodium phosphate (0.15 M). Aliquots of this cystamine solution were added to the cell suspensions to give final cystamine concentrations ranging from 0.5 to 5 mM. Aliquots of 5% cell suspensions were incubated for 60 or 120 min at 37° or 4° with or without cystamine. After incubation the cells were washed and resuspended in phosphate buffers. The final volume of the packed cells was 40% of the total. The mean corpuscular Hb concentration (MCHC) of control and cystamine-treated cells was determined from Hb and hematocrit measurements, except when stated otherwise. Intracellular pH was determined on packed frozen and thawed cells, at 37°, with a Radiometer pH meter fitted with a microelectrode unit (type E 5021a). Erythrocyte P₂-G level was determined according to Rose and Leibowitz (15). Hemolysates were prepared according to Drabkin (16). Hb electrophoresis was performed on cellulose acetate strips at pH 8.6 with Tris-EDTA-borate buffer (17); the proportions of the various fractions was determined by densitometry. Solutions of S-ethylamine Hb’s (cyst-Hbs) were prepared from cells that had been incubated with 2–5 mM cystamine for 1 hr at 37°. Organic phosphates and free cystamine were removed by chromatography on a column (2.5 × 40 cm) of Sephadex G-25.

Sickling Experiments. Aliquots (0.1 ml) of washed and packed cells were suspended in 10 ml of 0.15 M phosphate buffer at pH 7.45. The suspensions were subsequently deoxygenated by evacuation in a 250 ml tonometer at partial oxygen pressures of 0, 10, 20, 30, 40, or 50 mm Hg (0, 1.3, 2.6, 3.9, 5.2, or 6.5 kPa) for 10 min and incubated at 37°C for a further 10 min. Deoxygenated cells were then anaerobically transferred into a deoxygenated, phosphate-buffered glutaraldehyde solution (5% vol/vol) for fixation. The percentage of sickle cells was determined on at least 500 cells by phase contrast microscopy. We have designated sickle cells as cells that had sharp pointed projections, as well as deformed cells that were not biconcave discs.

Preparation of Fresh Reconstituted Cells with Different Mean Corpuscular Hb Concentrations. Fresh packed and washed erythrocytes (0.5 ml) were lysed in volumes of 0.05 M NaCl solution ranging from 0.4 to 1.5 ml. Careful mixing followed by incubation at 0° for 5 min was essential to ensure complete cell lysis. In some experiments, cystamine was added to the lysed cells for the preparation of cells containing equal proportions of Hb and cyst-Hb. Isotonicity was restored by addition of 1.3 M KCl solution. Cell preparations were subse-
quent incubation at 37° for 1 hr in order to allow rescaling; the cells were washed and finally suspended in the appropriate buffer. Control electrophoresis, oxygen affinity measurements, cellular \( P_2 - G \) and MCHC determinations were performed without delay, using a Coulter S or manual technique.

Oxygen Dissociation Studies. Oxygen dissociation curves were determined on erythrocytes and on Hb solutions at 37° by the spectrophotometric method of Benesch et al. (18), as modified by Bellingham and Huehns (19), with a Unicam SP 800. Oxygen dissociation curves of the erythrocytes were determined in 0.15 M phosphate buffers at pH 7.15 or 7.45. Oxygen equilibrium of 0.2% solutions of stripped Hbs and of cyst-Hbs was determined in 0.05 M Tris-HCl buffer in the pH range 7.15–7.45 or in 0.05 M [bis-(2-hydroxyethyl)aminomethyl]tris(hydroxymethyl)methane (Bis-Tris) at pH 6.45. The Bohr effect was determined from graphs relating the logarithm of the partial pressure of oxygen at which Hb is half saturated with oxygen (\( P_{50} \)) to pH. The interaction with \( P_2 - G \) was studied by determination of the \( P_{50} \) of stripped S-ethylamine and control Hb solutions, to which different concentrations of \( P_2 - G \) had been added.

Effect of Inositol Hexaphosphate (Ins-P6). The effect of Ins-P6 was studied by comparing the spectral changes undergone by the ferri form (MetHb) of Hb S and cyst-Hb S upon the addition of an excess of Ins-P6 (20). Hb S and cyst-Hb S were first oxidized to the corresponding MetHb in the presence of potassium ferricyanide and then chromatographed on a Sephadex G25 column equilibrated with 0.1 M sodium phosphate buffer (pH 6.5). Difference spectra were recorded in the absence and in the presence of a 5 molar excess of Ins-P6. 0.1 M phosphate buffer at pH 6.5 with a Cary 118 C spectrophotometer.

Gelation Studies. Gelation experiments were performed by the method of Singer and Singer (21), as modified by Bookchin and Nagel (22). Solutions of Hbs and of cyst-Hbs were subjected to simultaneous vacuum concentration and dialysis against 0.15 M potassium phosphate buffer (pH 7.35) at 4°. Hb S, cyst-Hb, and mixtures of Hbs S/cyst-S, S/A, cyst-S/A, S/cyst-A, and cyst-S/cyst-A were studied. All samples were subjected to control electrophoresis, MetHb measurements, and pH determinations before and after gelation. Samples containing more than 2% of MetHb (23) before, or more than 6% after, gelation were discarded. On account of the higher oxygen affinity of the cyst-Hbs, nitrogen was bubbled for 90 min; this period was sufficient to assure a complete deoxygenation of all the samples, as proven by recording the spectra.

Effect of Cystamine on Some Enzyme Activities and Substrates in Erythrocytes. The activities of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), hexokinase (EC 2.7.1.1), pyruvate kinase (EC 2.7.1.10), phosphofructokinase (EC 2.7.1.11), glutathione reductase (EC 1.6.4.2), and phosphoglycerate dehydrogenase (EC 1.2.1.12) and the concentration of ATP were determined in the cells treated with 1 mM cystamine and in control cells by the methods of Beutler (24).

RESULTS

The electrophoretic patterns obtained from hemolysates of A, SA, SC, and S cells that had been incubated for 1 hr with 1 mM cystamine are compared to those of controls (Fig. 1). A homogeneous, slowly migrating band was present in addition to the unreacted Hb in each incubated sample. This result was similar to those obtained with Hb solutions as previously described (13, 25, 26). It indicated that cystamine can penetrate the cell membrane and react with intracellular Hb. The reaction proceeded at identical rates for intracellular Hbs S, C, and A (Fig. 1). However, it was dependent on the cystamine concentration, the temperature, and the duration of incubation of the cells (Fig. 2). The incubation of the cells with 1 mM cystamine for 1 hr at 37° resulted, therefore, in the reaction of 55 ± 5% of the intracellular hemoglobin.

The mean corpuscular Hb concentration (MCHC) of A or S erythrocytes was constantly decreased when cells were incubated with cystamine in isotonic medium. The decrease in MCHC reached 5 ± 0.5% and 10 ± 1% at 1 mM and 2 mM cystamine concentrations, respectively, and was associated with an equivalent increase in the mean corpuscular volume.

Effect of Cystamine on Oxygen Dissociation Properties. The \( P_{50} \) values of S and A cells were studied before and after reaction with cystamine. The cystamine-treated S and A cells showed various degrees of increased oxygen affinity which depended on the extent of reaction of the intracellular Hb with cystamine. Reaction of about 55% of the intracellular Hb resulted in a 32% decrease in the \( P_{50} \) values of both A and S cells at pH 7.15 and 7.45 (Table 1). The \( P_2 - G \) levels and pH values, in controls and cystamine-treated cells, were identical. The increase in oxygen affinity of cystamine-treated cells could not therefore be explained by variations in \( P_2 - G \) concentrations or in intracellular pH.

The results of the oxygen dissociation properties of Cyst-Hbs A and S are shown in Table 2. The oxygen affinity of cyst-Hb S (or A) was considerably greater than that of unreacted Hbs without modification of the cooperativity. The Bohr effect over

![Table 1](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Cystamine (mM)</th>
<th>( P_{50} ) (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
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<td>0.5</td>
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</table>

![Table 2](https://example.com/table2.png)

<table>
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<th>Cystamine (mM)</th>
<th>( P_{50} ) (mm Hg)</th>
</tr>
</thead>
<tbody>
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<td>10</td>
</tr>
<tr>
<td>0.1</td>
<td>1.5</td>
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<tr>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

![Graph 1](https://example.com/graph1.png)

**Fig. 1.** Electrophoretic patterns of hemolysates from control cells and cells treated with 1 mM cystamine. (a, a') A cells; (b, b') SA cells; (c, c') SC cells; (d, d') S cells. Letters with primes indicate reacted cells. The incubation of the cells with 1 mM cystamine for 1 hr at 37° resulted in the reaction of 55 ± 5% of the intracellular hemoglobin.

**Fig. 2.** Effect of cystamine concentration on the rate of formation of cyst-Hb S in S erythrocytes. (○) Cells incubated for 2 hr at 37°; (O) cells incubated for 1 hr at 37°. Ranges include data from experiments on cells from 10 subjects.
Table 1. Effect of cystamine on the oxygen affinity of S and A cells ($P_{50}$)

<table>
<thead>
<tr>
<th>pH of buffers</th>
<th>A cells</th>
<th>S cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.15</td>
<td>Controls +1 mM cystamine*</td>
<td>Controls +1 mM cystamine†</td>
</tr>
<tr>
<td>31 ± 1.5</td>
<td>23–25</td>
<td>62 ± 5</td>
</tr>
<tr>
<td>24 ± 1</td>
<td>16–17.5</td>
<td>40 ± 2</td>
</tr>
</tbody>
</table>

Fifty-five plus or minus five percent of Hb was reacted with cystamine. Experiments were performed in 0.15 M phosphate buffer at 37°C.

* Three normal subjects.
† Seven patients.

the pH range 7.15–7.45 was reduced to −0.36 as compared to −0.50 for unreacted Hb S. These results obtained on solutions of cyst-Hb S are similar to those obtained by Taylor et al. (14) on solutions of cyst-Hb A. A lower interaction with $P_2$-G for cyst-Hb S (or A) was also found. The data in Table 2 indicate that the average effect of different concentrations of $P_2$-G on the shift towards the right of the oxygen dissociation curve of cyst-Hb S is 30% less than its effect on that of unreacted Hb S.

Effect of Cystamine on Sickling. At a partial oxygen pressure of 10 mm Hg, the cystamine-treated S cells showed a reduced tendency to sickle, which correlated with the cystamine concentration and hence with the proportion of the cystamine-reacted Hb (Fig. 3a). The percentage of sickle cells was reduced from 80% in untreated cells to 22% in cells treated with 1 mM cystamine, i.e., cells in which about 55% of the Hb was reacted. In cells treated with 2 mM cystamine, in which the reaction of intracellular Hb with cystamine was complete, erythrocyte sickling did not occur (Fig. 3a). However, even higher concentrations of cystamine (3 mM) did not apparently affect the irreversibly sickled cells, since their number in each sample (3–12%) remained unchanged.

Some inhibition of sickling by 1 mM cystamine occurred in completely deoxygenated cells. Only 60% of these cells were present in sickled form, as compared to 90% in preparations of unreacted cells. The effect on the inhibition of sickling by 1 mM cystamine was markedly enhanced when the oxygen pressure was raised from 0 to 20 mm Hg (Fig. 3b). This was expected from the high oxygen affinity of cyst-Hbs. The same data are shown in Fig. 3c, in which the abscissa represents the mean oxygen saturation of S cells from three patients. It clearly appears that the proportion of sickle cells is higher at a given oxygen saturation in the untreated samples than in the cystamine-reacted cells. These results indicate that a part of the anticolloidal effect of cystamine is independent of its influence on oxygen affinity.

Effect of Ins-$P_6$ on cyst-MetHb S. The addition of a 5 molar excess of Ins-$P_6$ to MetHb S in 0.1 M phosphate buffer at pH 6.5 produced spectral changes identical to those observed with solutions of MetHb A (20), and indicated conversion to the deoxy $T$ conformation. The addition of the same concentration of Ins-$P_6$ to cyst-MetHb S resulted in much smaller spectral changes than those obtained with MetHb S (Fig. 4), indicating a stabilization of the oxy $R$ conformation.

Dependence of the Oxygen Affinity of Cystamine-Treated Cells on MCHC. The dependence of the $P_{50}$ on the MCHC of control and cystamine-treated A and S cells is shown in Fig. 5. The cystamine-treated cells contained 55 ± 5% of their respective intracellular Hbs in a reacted form, as deduced from control electrophoresis. Unlike the A cells, the S cells displayed a great dependence of the $P_{50}$ upon MCHC. Their regression coefficient (0.95 mm Hg/g-dl) was higher than that of A cells.

Table 2. Effect of reaction of cystamine with Hb on the oxygen affinity ($P_{50}$) of hemoglobin S or A and on the $P_{2}$-G effect on $P_{50}$

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Molar ratio $P_2$-G/Hb</th>
<th>Hb S or A</th>
<th>cyst-Hb S or A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M Bis-Tris, pH 6.45, 37°C</td>
<td>0</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>0.05 M Tris·HCl, pH 7.45, 37°C</td>
<td>0</td>
<td>13.5</td>
<td>11</td>
</tr>
<tr>
<td>0.05 M Tris·HCl, pH 7.15, 37°C</td>
<td>0</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>4.5</td>
<td>32</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>42</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. (a) Effect of increasing concentrations of cystamine on sickling of S erythrocytes at a PO2 of 10 mm Hg. (b) Influence of oxygen pressure on sickling of control erythrocytes (solid line) and erythrocytes treated with 1 mM cystamine (dashed line). Sickling experiments were done in 0.15 M phosphate buffer, pH 7.45 at 37°C. Different symbols represent results obtained with cells from different patients. (c) Sickling is related to the hemoglobin oxygen saturation (same data as shown in b).

Fig. 4. Difference spectra between the original MetHb solutions (20 µM) in 0.1 M phosphate buffer (pH 6.5) and solutions at the same pH to which a 5 molar excess of Ins-$P_6$ had been added. (Continuous line) MetHb S; (dashed line) cyst-MetHb S; (dotted line) base line. Values of the extinction coefficient ε are in moles of heme.


**DISCUSSION**

The studies presented in this report provide evidence that cystamine, a thiol reagent, is a strong inhibitor of sickling. This agent appears to modify three interrelated processes that underlie the sickling of S erythrocytes: the Hb concentration within the S cells, their oxygen affinity, and the gelation of Hb S.

Cystamine reduced the MCHC slightly, but in a consistent manner. Its effect on membrane permeability may be due to the reaction of membrane SH groups with cystamine. The modifications in MCHC were not accompanied by any change in the intracellular pH nor in the intracellular concentration of organic phosphates which could affect oxygen affinity and sickling (27). The reduction in MCHC produces a delay in the sickling time, as demonstrated by kinetic studies of the gelation of deoxy Hb S (28) and of the sickling time of erythrocytes at zero oxygen pressure (29). The variation in MCHC seen in cystamine-treated S cells could be associated in a similar manner with a delay in sickling. The cystamine-treated cells exhibited a marked increase in their oxygen affinity. The part played in this increase by changes in the MCHC is probably small compared to the direct effect of cystamine on the oxygen affinity of Hb.

These modifications in the oxygen affinity of cystamine-reacted cells have to be compared to those observed with bis(N-maleimidomethyl) ether, an antisickling agent that crosslinks the cysteine β93 to the histidine δ97 (12). This substance increased the oxygen affinity but abolished the cooperativity of oxygen binding in the S cells. This result contrasts with the normal cooperativity of the cystamine-treated Hb. Such a discrepancy indicates that these two SH reagents may play a different role in the inhibition of sickling.

The slight but constantly increased minimum gelling concentration of reacted Hb S was in contrast to the absence of an effect by p-hydroxy mercuribenzoate. This reagent, which reacts with cysteine β93, raised the O₂ affinity for Hb but did not alter the minimum gelling concentration of Hb S or of a mixture of Hbs S and A (30). The modifications of minimum gelling concentration produced by cystamine suggest alterations in some bonds that form the polymer or gel. Moreover, since the mixtures of Hb S and cyst-Hb A have the same minimum gelling concentration as mixtures of Hb S and unreacted Hb A, it seems that Hb S itself must be altered. This supports a mechanism of conformational change that results from the stabilization of the quaternary oxy state and is suggested by the decreased interaction of cyst-Hb with P₅-G and by the attenuated spectral changes that occurred in the presence of excess Ins-P₅. This hypothesis is consistent with the conclusions of Bookchin and Nagel in studies of the minimum gelling concentration of half-liganded Hb S (31).

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**Table 3. Effect of cystamine on minimum gelling concentration in g/dl of Hb S and mixtures of Hbs A and S**

<table>
<thead>
<tr>
<th>% Hb S (α₃δ₂S)</th>
<th>% Cyst-Hb S (α₃δ₂S-cyst)</th>
<th>% Hb A (α₃δ₂A)</th>
<th>% Cyst-Hb A (α₃δ₂A-cyst)</th>
<th>MGC,*  mean value (g/dl)</th>
<th>Range</th>
<th>No. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>23.9</td>
<td>23.2–24.0</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>—</td>
<td>—</td>
<td>26.6</td>
<td>25.0–28.8</td>
<td>7</td>
</tr>
<tr>
<td>40</td>
<td>—</td>
<td>60</td>
<td>—</td>
<td>31.5</td>
<td>30.4–32.4</td>
<td>7</td>
</tr>
<tr>
<td>40</td>
<td>—</td>
<td>—</td>
<td>60</td>
<td>31.2</td>
<td>30.4–32.0</td>
<td>5</td>
</tr>
<tr>
<td>—</td>
<td>40</td>
<td>60</td>
<td>—</td>
<td>32.7</td>
<td>32.4–33.2</td>
<td>5</td>
</tr>
<tr>
<td>—</td>
<td>40</td>
<td>—</td>
<td>60</td>
<td>32.8</td>
<td>32.4–33.6</td>
<td>5</td>
</tr>
</tbody>
</table>

* Minimum gelling concentration.
Preliminary studies of the action of cystamine on erythrocyte enzyme activities revealed no major modification. However, extensive studies are required to determine the extent of the cellular alterations induced by cystamine. Preliminary findings in animals (32) and in healthy human subjects (33) have been reported, but careful toxicological studies have yet to be performed.

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