Methylisocyanate as an Antisickling Agent and Its Reaction
with Hemoglobin S*

CHOONG K. LEE
From the Center for Sickle Cell Disease and Department of Pediatrics and Child Health, College of
Medicine, Howard University, Washington, D. C. 20059

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Reviewing the reaction of potassium cyanate, an antisickling agent, with α-amino groups of
hemoglobin, it was found that the reaction was a slow process and requires a large excess of the reagent.
The reason for the slow reaction rate of carbamylation of hemoglobin by cyanate is that cyanate itself
does not react with hemoglobin. It is rather isocyanic acid, the reactive species, that reacts with
hemoglobin.

Since the pK of isocyanic acid is 3.8, only one out of 4,000 cyanate ions is present as isocyanic acid at
physiological pH. Therefore, it appears that a large excess of cyanate ions is required to achieve the
carbamylation of hemoglobin S, both in vivo and in vitro. Furthermore, the pH optimum for
carbamylation of carboxyhemoglobin and deoxyhemoglobin is 5.5 and, at pH 7.4, the reaction velocity
drops to one-third for carboxyhemoglobin and one-half for deoxyhemoglobin. To seek an approach to
reduce the dosage of cyanate and to increase the reaction velocity, an isocyanate derivative,
methylisocyanate which is already in the reactive form, was tested for its antisickling activity and its
reaction with hemoglobin S. It was found that methylisocyanate had antisickling activity and that only a
stoichiometric amount to 2-fold excess of the reagent over hemoglobin S α-amino groups was required to
prevent the sickling of erythrocytes. Methylisocyanate-treated sickle erythrocytes showed an increased
oxygen affinity compared to untreated. Methylisocyanate reacted with α-amino groups of hemoglobin S
and the reaction was complete in less than 1 min. Methylcarbamylated hemoglobin S had a higher
minimum gelling concentration than the untreated hemoglobin S. There was no detectable reaction of
free sulphydryl and α-amino groups of hemoglobin S with methylisocyanate. These results indicate that
methylisocyanate, and probably other isocyanate derivatives, possesses powerful antisickling activity.

Potassium cyanate was reported to have antisickling effect,
in vitro (1). It reacts with α-amino groups of hemoglobins S (1,
9). The carbamylated Hb S' has higher oxygen affinity than
the untreated and reduces the aggregation of Hb S (3). It was
found that 0.1 to 1.0 carbamyl group per hemoglobin tetramer
was required for this antisickling effect (1). Carbamylation of
Hb S at pH 7.4 and 37° with 400-fold excess of cyanate
introduced 1.5 carbamyl groups in 5 min. Isocyanic acid, the
reactive species of cyanate, was found to be a carbon dioxide
analog (2). However, carbon dioxide reacts with hemoglobin
much faster than cyanate does, in the range of milliseconds (4).
Since carbamylation of Hb S by cyanate is a slow process and
requires a large excess of reagent, cyanate administered to the
sickle cell patient can be transported to various organs of the
body during the reaction time. This leads to reaction with other
proteins of the body resulting in possible side effects. The low
reactivity of cyanate was examined in order to find a method to
increase the reaction velocity of the modification of α-amino

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'The abbreviations used are: Hb, hemoglobin; NaCl/PO₄, phos-
phate buffered saline, pHMB, β hydroxymercurobenzoate; MIH, 3
methyl-5-isopropylhydantoin.

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used for the α-amine group modification of Hb S, the reaction may proceed at a fast rate and would require only a smaller amount of the reagent to achieve carbamylation of Hb S. Based on this assumption, several isocyanate derivatives were considered for the tests of antisickling effect and reaction velocity.

Methylisocyanate was chosen as our example since it is soluble in water and is thought to be small enough to penetrate the cell membranes. Since alkylisocyanates are known to be hydrolyzed rapidly by water (7) and react with their amines to form urea derivatives, it was believed that only a small quantity of the added reagent would react with Hb S.

**EXPERIMENTAL PROCEDURE**

**Materials**—Methylisocyanate was purchased from Eastman Chemical Co. and glycyllysine hydrochloride from Calbiochem, respectively. Other reagents used were of reagent grade. Nucleopore polycarbonate filters (9.5 cm in diameter and 5 μm in pore size) were obtained from Nuclepore Corp., Pleasanton, Calif. Sickle blood samples were obtained from sickle cell patients at Howard University Hospital by venipuncture using Na_2EDTA or heparin as anticoagulant and refrigerated at 4°C until used. Blood samples used for experiments were those which demonstrated homozygous Hb S type by cellulose acetate electrophoresis at pH 8.6 in Tris/EDTA/borate buffer and contained less than 5% of Hb F by alkali denaturation method. The blood samples were used within 24 h after collection. Instruments used in these studies were Beckman 121 H amino acid analyzer, Instrumentation Laboratory model 217 blood gas laboratory system, Beckman ACTA M-VI spectrophotometer, American Optical series 20 microscope, and Radiometer pH-stat titration system with T112 titrator. Double-glass-distilled water was used throughout the experiments.

**Preparation of Standard 3-Methylisopropylhydantoin-Valine**—Sickle blood samples were washed three times with NaCl/Po_4, pH 7.4, 0.8, and centrifuged at 10,000 × g for 10 min. The original volume of the sample was reconstituted with NaCl/Po_4. The hemoglobin concentration of the samples was determined by cyanoethemoglobin method (9). Six milliliters of the washed red cell suspension were placed in a 80 ml glass vessel on ice and then incubated at 37°C, and incubated for 10 min with stirring at 37°C. The laboratory was kept free from cigarette smoke to prevent any binding of carbon monoxide by the samples. A stoichiometric amount or as much as a 2-fold excess of methylisocyanate over α-amino groups of Hb S (usually 2 to 5 μl) was added slowly from a Hamilton syringe (10 μl) to the stirring cell suspension and incubated for 10 min in the laboratory atmosphere. The red cells were then deoxygenated with hydrated 10% CO_2 in N_2 for 10 min. The sample at 37°C was diluted to 1% with 0.1 M KC_1 saturated with CO followed by 0.1 M NaOH. A 500-fold excess of methylisocyanate at pH 7.4 and 37°C for 10 min was dialyzed overnight in a Nucleopore polycarbonate filter (2.5 cm in diameter and 5 μm in pore size) under hydrostatic pressure. When 1 ml of the oxygenated suspension is passed through the filter, the filtrate collected was taken and subjected to the determination of the cell concentration along with the original sample. The absorbances of the cell suspensions at 540 nm were measured in a Double-glass spectrophotometer. The filtrates of deoxygenated samples, 5 ml each of the cell suspensions were first deoxygenated in a 80 ml glass vessel on ice with CO_2/KCl, treated with 10%, and centrifuged at pH 7.4. For each point of the oxygen saturation curve, the red cell suspensions were subjected to filtration on Nucleopore polycarbonate filter for the filtration of deoxygenated sample was very slow and virtually stopped after 0.8 ml of filtration, only 0.8 ml of the filtrate was used for the absorbance measurement at 540 nm. The ratio of the absorbances of filtrates and original samples were calculated to obtain a determination of each sample.

**Preparation of Standard 3-Methylisopropylhydantoin-Valine**—Sickle blood samples were washed three times with NaCl/Po_4 or dialyzed against 500 volumes of NaCl/Po_4 at 4°C. The sample was treated similarly. Deoxygenation, fixation, and counting of the cells were performed as described earlier. Carbonmonoxo Hb S in 0.1 M KCl which had 1 N-methylcarbamyl residue at NH_2-terminal groups was dialyzed overnight against 500 volumes of 0.1 M KCl saturated with CO at 4°C. After the determination of the protein concentration, the sample was subjected to MH assay. The result was compared with the value of the undialyzed. There was no valine detected in any of the samples from cigarette smoke.

**Preparation of Standard 3-Methyl-5-isopropylhydantoin—Valine** (20 mg) was dissolved in 2 ml of water in a 15 ml ignition tube. The pH was adjusted to 3.0 with 1 N NaOH. A 500-fold excess of methylisocyanate was added to the valine solution with stirring. The mixture was kept in a well-ventilated hood overnight at room temperature to synthesize N-methylcarbamylhydantoin. Glacial CH_3COOH (2 ml) and 12 N HCl (4 ml) were added to the reaction mixture and the tube was heat-sealed. The mixture was incubated at 100°C for 1 h to cyclize N-methylcarbamylhydantoin to 3-methyl-5-isopropylhydantoin. The mixture was then dialyzed against 500 volumes of 0.1 M KCl in a Nucleopore polycarbonate filter at 48°C for 48 h. The filtrate was then subjected to MH assay to determine the compound accurately. Element analysis was not conducted in order to bypass the difficulty of weighing this hygroscopic compound. The filtrate was then subjected to MH assay to determine the compound accurately. Element analysis was not conducted in order to bypass the difficulty of weighing this hygroscopic compound.
rotary evaporator and dissolved to 2 ml with water. Aliquots of this solution were subjected to column chromatography, alkaline hydrolysis, and amino acid analysis as described later in the text.

**Determination of N-Methylcarbamylation of Amino Groups of Hb S**—The extent of reaction between methylisocyanate and α-amino groups of Hb S was determined by employing hydantoin assay (14) with minor modifications. N-Methylcarbamylation of the NH₂-terminal group of Hb S, valine, and its cyclization in 6 N HCl at 100° for 1 h yielded MIH. Hydrolysis of standard MIH with 0.2 N NaOH at 100° for 22 h in a sealed tube (14) gave 22% recovery of valine based on amino acid analysis. Incubation of MIH with 0.4 N NaOH at 110° for 24, 48, and 72 h yielded 74, 88, and 90% recovery of valine, respectively. Concentration of NaOH higher than 0.8 N formed excessive silica particles in the tube, which prevented the accurate assay of valine. When MIH was passed through a Bio-Rad AG 50 W-X2 column (0.9 x 10 cm) and the effluent was monitored with absorbance at 225 nm, considerable trailing was observed. The first 30 ml of the effluent was collected, evaporated to dryness, and hydrolyzed with 0.4 N NaOH for 48 h at 110°. Based on the amino acid analysis of the above sample and the control sample, which was not passed through the column, it was found that 82% of the compound was recovered after the column chromatography. This recovery value and another value obtained after the hydrolysis of MIH with 0.4 N NaOH for 48 h were used for the calculation of the extent of the reaction between Hb S and methylisocyanate. For the determination of the reaction extent of α-amino groups of Hb S with methylisocyanate, N'-methylcarbamyllysinine was prepared in the following manner: 1 mmol of glycyllysine hydrochloride in 1 ml of water was mixed with a 300-fold excess of methylisocyanate in a well-ventilated hood and incubated overnight at room temperature. The solution was adjusted to 6 N HCl using 12 N HCl, and incubated at 100° for 1 h in a sealed ignition tube. The mixture was evaporated to dryness and subjected to amino acid analysis in a 0.2 N citrate buffer, pH 3.49. The N'-methylcarbamyllysine was eluted between alanine and valine. Methylisocyanate-treated Hb S was hydrolyzed with 6 N HCl, in vacuo, at 110° for 22 h and subjected to amino acid analysis to determine any modification of the α-amino groups.

**Reaction Rate of N-Methylcarbamylation of Hb S α-Amino Groups by Methylisocyanate**—Since alkylisocyanates are known to decompose rapidly in water (7) and the decomposition rate of methylisocyanate is not known, the second order rate constant of N-methylcarbamylation of Hb S α-amino groups by methylisocyanate was not determined. The reaction rate was monitored by MIH assay. Six milliliters of dialyzed carbonmonoxy Hb S solution (0.8 mm M KCl) were equilibrated at 37° in a reaction vessel of pH stat system, TTT2 of Radiometer. The pH was adjusted to 7.4 with 0.2 N NaOH. Two-fold excess of methylisocyanate over Hb S α-amino groups was added to the Hb S solution with a Hamilton syringe (10 μl) to initiate the reaction. Titrant used was 0.2 N NaOH. Aliquots of the reaction mixture (0.5 ml) were removed at 15, 30, and 45 s and 1.5- and 2-min intervals and placed in 5 ml of ice-cold 5% trichloroacetic acid to terminate the reaction. The precipitated hemoglobin was washed five times with 5 ml of ice-cold 5% trichloroacetic acid solution and two times with ethyl ether. The precipitate was dried in a warm water bath and then dissolved in 5 ml of 50% acetic acid. The product was subjected to the determination of N-methylcarbamylation of Hb S α-amino groups by the procedure given above.

**RESULTS**

**Effect of Methylisocyanate on the Sickling of Erythrocytes**—Deoxygenated red cells (Hb S type) are shown in Fig. 1. Most of the red cells in the control sample were in sickle shape (Fig. 1a). With 2-fold excess of methylisocyanate over Hb S α-amino groups, most of the cells retained the normal morphology upon deoxygenation (Fig. 1b). No hemolysis of red cells was observed in this experiment. Methylisocyanate did not reverse the sickling of irreversibly sickled cells. Increasing the amount of methylisocyanate, there was an increase of the number of N-methylcarbamyl groups incorporated into Hb S with a concomitant decrease of the number of sickle-shaped cells (Table I).

**Determination of Minimum Gelling Concentration**—The minimum gelling concentrations of control and methylisocyanate-treated Hb S are shown in Table II. The minimum gelling concentration of the control Hb S was 24.0 ± 0.0 g/100 ml, which is comparable with published data (10). Treatment of Hb S samples with methylisocyanate increased the minimum gelling concentration up to 31.5 ± 0.9 g/100 ml, suggesting that N-methylcarbamylation of Hb S reduces the aggregation of Hb S molecules.

**Effect of Methylisocyanate on Oxygen Affinity of Hemoglobin S**—Fig. 2 shows oxygen dissociation curves of control and N-methylcarbamylated red cells, which has 0.4 N-methylcarbamyl residues at Hb S α-amino groups, for the determination
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Sickle cells were washed three times with NaCl/PO₄ and the original volume was reconstituted with NaCl/PO₄. Hemoglobin concentration was 1.3 μM. Incorporation of N-methylcarbamyl group to the α-amino groups of hemoglobin S was determined by modified hydantoin assay as described in the text.

<table>
<thead>
<tr>
<th>[CH₄NCO]/[Hb α-NH₂]</th>
<th>Number of N-methylcarbamyl groups incorporated per Hb S tetramer</th>
<th>% sickle cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>87.6</td>
</tr>
<tr>
<td>1.0</td>
<td>0.4</td>
<td>47.5</td>
</tr>
<tr>
<td>1.5</td>
<td>0.6</td>
<td>34.1</td>
</tr>
<tr>
<td>2.0</td>
<td>0.8</td>
<td>27.2</td>
</tr>
</tbody>
</table>

Test for Filterability of Sickle Cells—Filtration of control and methylisocyanate-treated samples in the oxygenated condition gave filtrates which had the same concentration of red cells as in the original samples. The filtrate of the deoxygenated control sample contained 0.0% of the red cells. Filtration of the treated cells in the deoxygenated condition resulted in permeation of 80.2 ± 5.8% of the cells through the polycarbonate filter in the given experimental condition. These results indicate that the membrane deformability of the sickle cells were restored to a great extent by N-methylcarbamylation.

Test for Irreversible Antisickling Effect of Methylisocyanate—The washed or dialyzed red cells previously treated with methylisocyanate retained their normal morphology upon deoxygenation. The MIH assay of a Hb S sample, which had one N-methylcarbamyl group of its α-amino groups, prior to and after dialysis gave identical results suggesting that the dialysis did not remove the N-methylcarbamyl groups from Hb S. These observations indicate that the antisickling effect of methylisocyanate is permanent and that there was a stable bond formation between Hb S α-amino groups and methylisocyanate, like in the case of potassium cyanate (1).

Determination of Free Sulfhydryl Groups of N-Methylcarbamylated Hb S—The titratable free sulfhydryl groups in methylisocyanate-treated and control Hb S sample were 1.7 and 1.6 residues/hemoglobin tetramer, respectively. These results suggest that there was no stable bond formation between Hb S α-amino groups and methylisocyanate.

Test for Modification of Hb S α-Amino Groups by Methylisocyanate—Amino acid analysis of the methylisocyanate-treated Hb S hydrolysate showed no detectable N-methylcarbamylsine, indicating that there was no modification of Hb S α-amino groups by methylisocyanate.

Reaction Rate of N-Methylcarbamylation of Hb S by Methylisocyanate—Carbonmonoxy Hb S treated with 2-fold excess methylisocyanate over Hb S α-amino groups (Fig. 3) showed that the reaction was complete within 46 s after the initiation, and that one N-methylcarbamyl group was introduced per Hb S molecule. This incorporation was at the α-amino groups of Hb S. After the addition of 3.0 μl of methylisocyanate (2-fold excess over α-amino groups) to the carbonmonoxy Hb S in the given experimental condition, a continuous decrease in the pH was observed. The reason for the pH drop in the dilute hemoglobin solution is probably due to carbonic acid formation with CO₂, a decomposition product of methylisocyanate. Some of the other decomposition product, methylamine, could escape to the air since the unprotonated form of methylamine has a boiling point of −6°C. The pH decreased to 7.2 at 37°C after the addition of methylisocyanate and remained constant, suggesting that the reaction was stopped at this point. This observation supports the data obtained by amino acid analysis.

Discussion

The discovery of potassium cyanate as an antisickling agent (1) offered a new approach for the treatment of sickle cell disease and the understanding of the Hb S molecule. The basis for the antisickling effect of potassium cyanate was found to be mainly due to the carbamylation of the Hb S α-amino groups (3). The α-amino groups of α chains are known to contribute about 25% to the alkaline Bohr effect (15). Carbamylation of...
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these groups reduces the alkaline Bohr effect, thereby increasing the oxygen affinity of the Hb S molecule. Oxygenated Hb S molecules do not aggregate and thus prevent the sickling of erythrocytes. The carbamylation of the \( \beta \) chain \( \alpha \)-amino groups, \textit{in vivo}, was 2 times less frequent than that of \( \alpha \) chain (16). Therefore, the contribution of the \( \beta \) chain carbamylation in the antisickling effect would be relatively small. However, the \( \beta \) chain-carbamylated Hb S showed about 23\% increase in minimum gelling concentration (3). Since the antisickling effect of potassium cyanate is due to the modification of the Hb S \( \alpha \)-amino groups, it was desirable to find a reagent which reacts fast with Hb S and requires a smaller amount of the reagent to achieve the modification. Methylisocyanate, one of the isocyanate derivatives which are in reactive form, was investigated as an example for the above purpose. It was found that this reagent has powerful antisickling activity and reacts fast with Hb S \( \alpha \)-amino groups. The antisickling activity of methylisocyanate was irreversible, like that of potassium cyanate (1). Since there were increases of oxygen affinity of the sickle cells and minimum gelling concentration of Hb S after incubation with methylisocyanate, it is believed that the reagent reacted with both \( \alpha \) and \( \beta \) chain NH\(_2\)-terminal as cyanate does. Since methylisocyanate is an analog of isocyanic acid, the antisickling effect of methylisocyanate appears to be similar to that of potassium or sodium cyanate (1, 3) in many respects. The difference between methylisocyanate and potassium or sodium cyanate is that smaller amount of methylisocyanate is required for the antisickling effect and methylisocyanate possesses a higher reactivity than potassium or sodium cyanate. Since methylisocyanate is a lachrymator, it may not be advisable for use \textit{in vivo}. The use of this reagent \textit{in vitro} without an additional process, like dialysis, may depend on the toxicity of methylamine and 1,3-dimethylurea in a methylisocyanate-treated medium. Methylisocyanate will decompose to methylamine in an aqueous solution. Methylamine could react with methylisocyanate to form 1,3-dimethylurea. Any possible side effects by these byproducts in the red cell suspension after reinfusion have yet to be investigated. Should the concentrations of methylamine and 1,3-dimethylurea in methylisocyanate-treated sickle cell suspension be so high as to result in side effects in the sickle cell patient, then these byproducts should be removed from the red cell suspension, either by washing or by dialysis before the reinfusion. Methylamine has a boiling point of -6\(^\circ\) and pK of 10.6. The evaporation rate of methylamine in the red cell suspension at pH 7.4 and 37\(^\circ\) is currently under investigation in this laboratory.

Isocyanic acid, the reactive tautomer of cyanate, was found to be a carbon dioxide analog (2), and the specificity of isocyanic acid for \( \alpha \)-amino groups of hemoglobin was explained on the basis of the structural analogy between carbon dioxide and isocyanic acid. This report shows that the antisickling reagents need not be analogs of CO\(_2\) in order to react with \( \alpha \)-amino groups of Hb S and to prevent sickling of erythrocytes. Methylisocyanate, which is not a structural analog of carbon dioxide, reacted with \( \alpha \)-amino groups of hemoglobin by virtue of its reactivity. At this point, the scope of screening new antisickling reagents broadens.

It would be of great interest to find reactive compounds which are small enough to penetrate the erythrocyte membrane and react with Hb S \( \alpha \)-amino groups. Other isocyanate derivatives may also react fast with Hb S \( \alpha \)-amino groups, and may, if they can penetrate the erythrocyte membrane, possess antisickling activity.

The structural and elemental analysis of the product of the reaction of methylisocyanate with valine followed by cyclization has not been performed at this time. However, the chemistry of hydantoin synthesis (13) suggests that the compound is most likely to be 3-methyl-5-isopropylhydantoin. The preparation of pure MIH was difficult because of its extremely high hygroscopic nature. It was also difficult to hydrolyze MIH to valine because of the stabilizing effect on the hydantoin ring by the methyl group at position-3. We found that methylisocyanate reacted with several amino acids and may be used for the determination of NH\(_2\)-terminal groups of proteins.

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C K Lee


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