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Solubility Changes Observed in Sickle Cell Hemoglobin as the Amino Groups are Carbamylated (38321)

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Sickle cell anemia is one of the best understood of the molecular diseases of man. The individual who is afflicted with sickle cell disease is homozygous in the gene for hemoglobin S (Hb S). Hb S differs from normal adult hemoglobin in that the glutamyl residue normally at position 6 from the *N*-terminous of the beta chain has been replaced by a valine residue. The cause of erythrocyte sickling is thought to be the result of the polymerization of deoxyhemoglobin S molecules to form tactoids. The exact mechanism of polymerization remains to be elucidated. These tactoids distort the red blood cell into bizarre shapes which cause vasocclusive episodes, clinically referred to as "crises." Recently investigators have attempted to show that certain chemical agents are reliable means to either alleviate or prevent a crisis from occurring.

In this latter case, cyanate, has been shown to inhibit the sickling of erythrocytes *in vitro* (1, 2) but not *in vivo*. Cyanate reacts with hemoglobin to form a covalent bond with the alpha amino groups and with the epsilon amino groups in a reaction called carbamylation. A detailed kinetic study of this reaction has been completed by Lee and Manning (3). Their results show that at five minutes of reaction time only alpha amino groups have reacted with the cyanate. At 30 min some nonspecific carbamylation of the epsilon amino groups has occurred and at longer reaction times more of the epsilon amino groups are carbamylated. Under these conditions of excess cyanate it is obvious that the site of reaction is fairly nonspecific. The purpose of this study was to investigate the solubility of hemoglobin S with progressive carbamylation of the molecule.

Materials and Methods. Preparation of hemoglobin. Whole blood was obtained from individuals with normal hemoglobin A, sickle cell trait, heterozygotes for hemoglobins A and S, and sickle cell disease homozygotes for hemoglobin S by venipuncture. Ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant. The whole blood was centrifuged at 1000*g* for 10 min. The packed erythrocytes were washed three times with 0.9% saline and lysed by adding an equal volume of distilled water to the packed red blood cells. The mixture was shaken vigorously for two minutes and centrifuged at 10,000*g* for 30 min. The clear lysate was removed and dialysed against 0.1 *M* sodium phosphate buffer at a pH=7.4 for 16 hr at 4°. The concentration of the hemoglobin sample was determined by the cyanmethemoglobin method (4) at 540 nm. Samples were routinely purified by chromatography on a carboxymethyl-Sephadex column as described by Dozy and Huisman (5) and the purified fractions were concentrated by vacuum dialysis at 4°. In some experiments the purified hemoglobins A and S were used, in others mixtures of Hemoglobin A and S were used as obtained from the donor heterozygous for these hemoglobins without purification.

Carbamylation. Potassium cyanate was added to oxyhemoglobin solutions to a concentration of 0.01–0.25 *M*. These solutions were incubated at 37° and gently shaken during the course of the reaction. The final concentration of the hemoglobin was 50 mg/ml. Aliquots were removed periodically from the reaction flasks for the determination of the hemoglobin solubility, molar ratio of cyanate incorporation to hemoglobin, and cellulose acetate electrophoresis.

Hemoglobin solubility. A slightly modified method of Itano (6) was followed. Eight milliliters of 2.8 *M* Phosphate buffer containing 10 mg per ml sodium dithionite, $Na_2S_2O_4$, was added to a 15 ml Corex test tube. 1 ml of distilled water was carefully layered over the top of the buffer

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and 1 ml of the hemoglobin reaction mixture was added. The tube contents were shaken and allowed to stand at room temperature for 30 min then centrifuged at 10,000*g* for 20 min and the supernatant was removed by aspiration into a syringe. Duplicate aliquots of this supernatant were used to determine the concentration of the hemoglobin remaining in solution.

Cellulose acetate electrophoresis. Hemoglobin electrophoresis was performed on the electrophoretic apparatus described by Barnes *et al.* (7) with the modification of Koenig *et al.* (8). A sample of the hemoglobin reaction mixture was applied with a #3 sable hair paint brush onto the cellulose acetate strips and electrophoresed at 200 V for 10 min. Following electrophoresis the cellulose acetate was stained with Ponceau S for 2 min and destained with three washes of 2% acetic acid.

^{14}C-Cyanate incorporation. Aliquots of the hemoglobin mixture (0.5 ml) were chromatographed on a Sephadex G-25 column to separate the hemoglobin from the unbound cyanate. The column dimensions were 0.9 × 30 cm. A 0.5 ml aliquot was removed from the pooled hemoglobin fraction and added to a scintillation vial. To this we added an additional 0.5 ml of 30% H_2O_2 to bleach the hemoglobin as described by Jensen *et al.* (9). At least 30 min were allowed to pass and then 10 ml of aquasol was added. The vials were placed in a Beckman LS 2333 liquid scintillation counter allowed to stand for several hours before counting. Another 0.5 ml aliquot was used to determine the concentration of hemoglobin.

Results. Carbamylation has the overall effect of lowering the solubility of hemoglobin. Tabulated in Table I is the result of a typical experiment using hemoglobin from a patient heterozygous for hemoglobins A and S. Examination of these results suggests a slight increase in solubility in 0.05 *M* and 0.10 *M* potassium cyanate at 30 min of incubation. At 60 and 90 min of incubation there was a large reduction in the solubility of the hemoglobin.

In Fig. 1 this decrease in solubility of hemoglobin is shown for purified hemoglobin A, S and the mixture of A and S. All three systems appear to behave similarly. With increasing carbamylation there is sharp reduction in the solubility of the hemoglobin regardless of whether the hemoglobins have been purified, 1a and 1c, or as the mixture of A and S, 1b.

In Table II we have shown the number of moles of cyanate which have reacted with a mole of hemoglobin S at certain specific time intervals. The apparent two-fold difference of our result from that of Lee and Manning (4) is probably due to different experimental conditions.

An example of how the cellulose acetate electrophoresis pattern changes is shown in Fig. 2. The control sample, a hemoglobin A/S mixture has the two expected electrophoretic bands. However, even at 10 min incubation with the potassium cyanate the discrete bands have merged into a continuum. At longer incubation times there are no distinguishable bands at the higher cyanate concentrations.

Discussion. Carbamylation of hemoglobin S has been shown to inhibit the sickling of red blood cells *in vitro* (1, 2). This observation led us to investigate a plausible reason for this apparent protection against sickling. Namely, a suspected change in the solubility of the hemoglobins involved. We have, however, demonstrated that instead of greatly increasing the solubility of hemoglobin S which would perhaps prevent the hemoglobin from aggregating, in fact we see a rather dramatic decrease in solubility at the longer reaction times. The data in Table I does show a slight increase in solubility but perhaps more importantly there is this precipitous decrease in solubility noted previously. Lee and Manning (3) have shown that at five minutes of

TABLE I. Hemoglobin Solubility at Different Cyanate Concentrations.*

Cyanate Concentration	Time in minutes			
	0	30	60	90
0.00*M*	1.30	1.31	1.35	1.33
0.05*M*	1.27	1.48	1.35	1.22
0.10*M*	1.26	1.35 1.23	1.12	0.25*M*
1.20	1.20	1.00	0.81	

* mg of hemoglobin/ml remaining in solution.

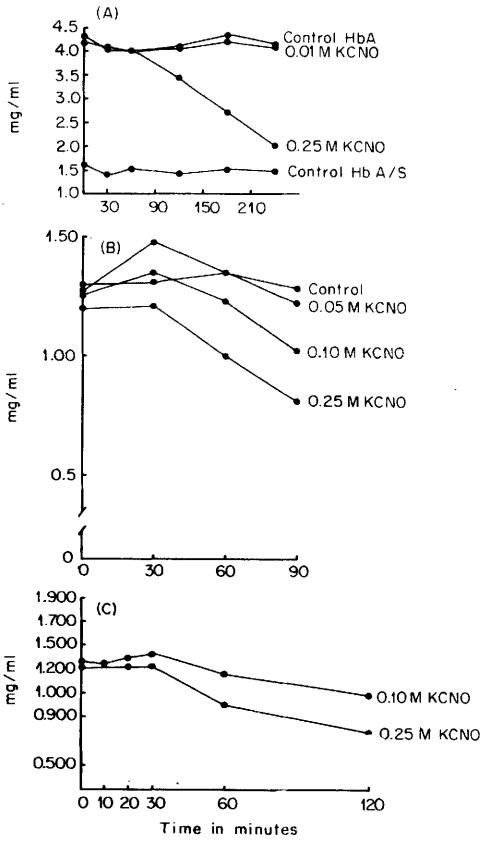

FIG. 1. Effect of potassium cyanate on the solubility of hemoglobins A, S and a mixture of A and S. (A) Solubility of HbA in 2.24 *M* phosphate buffer. Control samples contain no potassium cyanate. (B) Solubility of hemoglobin from a patient heterozygous for hemoglobins A and S in 2.24 *M* phosphate buffer. (C) Solubility of purified hemoglobin S in 1.96 *M* phosphate buffer.

reacting an excess of sodium cyanate at pH=7.4 the predominate site of carbamylation is the alpha amino groups of hemoglobin. Our experimental results suggest hat at this reaction time of 5–10 min there is a slight increase in the solubility of the hemoglobin at the higher concentrations of cyanate.

By 30 min when Lee and Manning show about 3 of the *N*-terminal valine and two lysine residues carbamylated the hemoglobin is already less soluble. As carbamylation proceeds the hemoglobin becomes less soluble; therefore the observed protection observed for the red blood cells *in vitro* can not be due to an increase in the solubility of the hemoglobin but is perhaps a result of hindering the ability of the hemoglobin to form the long tactoids needed to distort the shape of the red blood cell.

Carbamylation of the amino groups causes a charge change on the hemoglobin molecule. Because of this electrophoresis is a useful tool to monitor the progress of this reaction. If carbamylation occurs at a limited number of specific sites, such as the *N*-terminal valines, then the electrophetic pattern expected would be the formation of discrete bands. However, as carbamy-

TABLE II. Carbamylation of Hemoglobin S.*

Time in minutes	0.25*M*	Lee (4)
30	11	6
60	16.4	9
120	24.3	10.5
240	32	17

* Mole of cyanate/mole of hemoglobin.

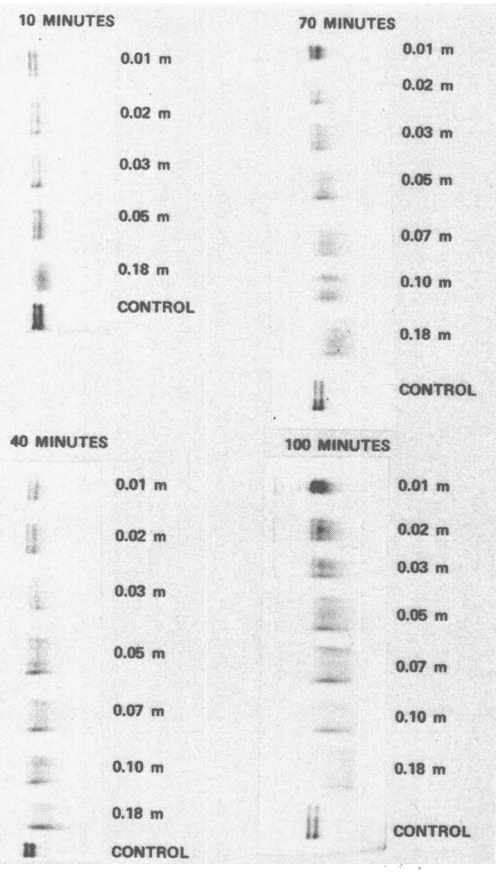

FIG. 2. Electrophoresis of a hemoglobin A/S mixture on cellulose acetate which has been stained with Ponceau S. At the higher concentrations of cyanate and longer incubation times the discreteness of the bands disappear and become a continuum.

lation occurs the electrophoresis pattern became a continuum, a complete loss of distinguishable bands, thus suggesting that carbamylation occurs nonspecifically on the hemoglobin under the conditions used in our laboratory.

Summary. The alpha and epsilon amino groups of oxyhemoglobin A, S, and A/S mixture have been carbamylated and the solubility of these hemoglobins have been monitored during the time course of this reaction. Initially, the solubility of the hemoglobin increases slightly with carbamylation; however, this change is reversed, and the hemoglobin becomes much less soluble as more amino groups react with the cyanate. This change in solubility of hemoglobin is dependent upon both the cyanate concentration and the time of reaction. We have used the Itano (6) solubility test to measure the change in the solubility of these chemically modified hemoglobins. This test is a potent tool for monitoring changes in solubility in the course of chemical modification of hemoglobins.

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