Urea, Urease, Cyanate, and the Sickling of Hemoglobin S


Urea in glucose solutions has been advanced as a chemotherapeutic agent in sickle cell disease because it has been found effective both in reversing and in blocking sickling. It has been suggested recently that this beneficial action of urea may be the result of formation of cyanate from urea and subsequent carbamylation of beta S globin chains in the hemoglobin S molecule. In this paper, we show that the urea molecule per se interferes with sickling. SS cells in oxygen-free atmosphere, protected from sickling by urea-sugar solutions, were sickled after urease was added. Urea blocks and reverses sickling; urea-protected sickle cells become sickled after incubation with urease; these effects are reversible and immediate, and are apparent in the deoxygenated state. In contrast, cyanate blocks but does not reverse sickling; cyanate-treated cells are unaffected by urease; these effects are permanent and require time; and hemoglobin S must first be oxygenated before carbamylation will occur. Evidently, urea and cyanate interfere with sickling by distinctly separate molecular mechanisms. Possible mechanisms for the action of urea are discussed.

Method

A specimen of fresh intact, homozygous S red cells was divided into two aliquots of 0.2 ml each and delivered into each of two 10 × 75 mm tubes. One aliquot served as a control while the other specimen was treated experimentally.

Aqueous glucose solution, 0.2 ml of a 50 g/liter, was added to the control tube, to give a final glucose concentration of 25 g/liter in the control blood–glucose system. Two-tenths milliliter of the same glucose solution (50 g/liter) containing 100.0 mmol of urea per liter was added to the experimental tube, producing a final urea concentration of 50 mmol/liter (equivalent to a blood urea nitrogen (BUN) of 140 mg/100 ml).

Both tubes were then deoxygenated for 1 h in a constant temperature bath at 37.0°C as follows. Each tube was fitted with a rubber stopper previously pierced by one 20-gauge and one 22-gauge needle. To the former needle, rubber tubing was connected leading from a nitrogen gas source. The nitrogen was bubbled at a constant flow rate of 2.5 liters/min through water traps to moisten the gas going to the blood–glucose systems. The 22-gauge needle served as an exhaust vent.

After deoxygenation the tubes were placed, with stoppers still intact, into an oxygen-free glove-bag inflated with nitrogen gas. Under these anoxic conditions, wet slides of the specimens from each tube were prepared and sealed with fingernail polish. While still in the oxygen-free atmosphere in the glove-bag, 0.2 ml of aqueous glucose solution (25 g/liter) was added to the control tube to maintain isoncoticity; to the experimental tube 250 units of urease (type C-3; Sigma Chemical Co., St. Louis, Mo. 63178) in 0.2 ml of aqueous glucose (25 g/liter) was added.

Once again, the tubes were deoxygenated as before, for 2.5 to 4.0 h, at 37.0°C. Both tubes were then placed in the oxygen-free glove-bag as before, and wet slide preparations were made of specimens from each tube. The slides were sealed with fingernail polish under anoxic conditions and counted.

Results

By use of the methods described, in three consecutive cases of homozygous S erythrocytes (kept in oxygen-free atmospheres throughout),
erythrocytes were sickled by exposure to nitrogen (Figure 1) in the control specimen.

In the experimental tube, deoxygenation by nitrogen in the presence of urea as described restored and (or) maintained erythrocytes in their normal conformation (Figure 2). The subsequent addition of urease and incubation at 37°C resulted in the restoration of sickling (Figure 3). These results were consistently reproducible with homozygous S cells from three different patients (Table 1).

Discussion

The deduction from molecular theory that urea should be an effective chemotherapeutic agent in sickle cell disease has been supported by a growing body of experimental and clinical evidence (1–7). Urea in sugar solutions has been shown to reverse (restore sickled cells to the original normal configuration) and to block (prevent sickling under conditions otherwise conducive to sickling) sickling. Hence, the molecular mechanism of the action of urea in these blocking and desickling reactions with hemoglobin S are of substantial interest.

Murayama has shown that concentrated, deoxygenated hemoglobin S hemolysates in the gel state, i.e., “sickled,” can be dispersed by (a) lowering the temperature of the system (8–11), (b) increasing the hydrostatic pressure (12), and (c) by introducing short-chain aliphatic hydrocarbons such as propane under low pressure into the system (10, 11, 13). On the basis of this and other evidence, Murayama developed a molecular hypothesis for the mechanism of sickling (10, 13), which he has recently modified (3). The modified Murayama hypothesis implies that a chemical method that prevents or destroys the formation and perpetuation of the pathologic inter-tetrameric hydrophobic bonds leading to the sickling event may be of therapeutic value in preventing or reversing sickling (2–6).

Such a chemotherapeutic agent obviously should (a) be water soluble, (b) be nontoxic, (c) be easily diffusible through body fluids, (d) traverse the red blood cell membrane without injurious sequelae, and (e) be able to break hydrophobic bonds to a modest degree without denaturation of the tertiary structure of the quaternary structure of the hemoglobin molecule (2–5). This latter requirement implies a cluster or linear array of hydrogen groups at some point on the candidate molecule. Bruning and Holtzer (14) showed that urea could break hydrophobic bonds in addition to its well-known action of breaking hydrogen bonds as originally proposed by Mirsky and Pauling in 1936 (15). This finding was quickly confirmed (16–17). Hence, on this evidence and for these reasons, urea was selected as a chemotherapeutic agent in the treatment of sickled hemoglobin S erythrocytes. Urea, in concentrations as low as 6 mmol/liter, has a blocking effect on sickling (2). Furthermore, we have aborted sickle cell crises by reversal of sickling by BUN concentrations of 150–200 mg/100 ml [about 53.5–71.2 mmoles of urea per liter (3–5)].
Table 1. Effects of Nitrogen, Urea, and Urease on Sickling and Desickling of SS Erythrocytes from Three Patients

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Percent of cells sickled under N₂ before urea treatment</th>
<th>Percent of cells sickled under N₂ after addition of urea</th>
<th>Percent of cells sickled under N₂ after addition of urease and incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>96</td>
<td>7</td>
<td>94</td>
</tr>
<tr>
<td>SS</td>
<td>93</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>SS</td>
<td>68</td>
<td>6</td>
<td>67</td>
</tr>
</tbody>
</table>

At least 500 erythrocytes were counted to determine the percentage of sickling. A plausible explanation of the somewhat lower but consistent levels of sickling in the last specimen may be a reduced concentration of the Murayama-Hasegawa sickling co-factor (S).

The molecular mechanism by which urea acts in this manner is of great interest. At least four possible mechanisms may be suggested on the basis of available information:

1. Urea may form a clathrate around the hydrophobic substituted number six valine residue in each beta S globin chain, or urea may combine (urea-to-valine) at such points on the hemoglobin S tetramer. By such reactions the conformation of the hemoglobin S molecule is altered and sickling is prevented or reversed by steric hindrance, a therapeutic principle implied in the modified Murayama hypothesis (2, 3).

2. Second, because urea has an electric dipole moment of 4.6 compared to a value of 1.8 for the water molecule, it is possible that urea molecules may disperse and randomize the precisely structured arrangement of water molecules required in the formation and maintenance of the pathologic hydrophobic bond for polymerizing the molecules of hemoglobin S.

3. Third, both of the above mechanisms may be involved. On the basis of available information these views can only be presented as hypotheses, and considerable rigorous study of the problem by physical chemists will be required before the mechanisms at work are understood.

4. A fourth possible explanation for the molecular action of urea in its interference with the sickling event has been recently suggested by Cerami and Manning (18). Urea ionizes to a very small extent in aqueous solutions to form cyanate ions, and these ions may carbamylate the N-terminal valine amino residues (the natural number one valine residue and not the substituted pathological number six valine residue) in about 25% of the beta S globin chains. This suggestion has already been commented upon (18, 20). There is no doubt that, in vitro, carbamylation with the hemoglobin S molecule does occur in the presence of potassium cyanate. However, when the modes of action between urea and potassium cyanate are compared (Table 2) it is clear that these two compounds, urea and potassium cyanate, act in a separate, unrelated manner at the molecular level (21). Our experiments with urease indicate that the urea molecule per se mediates the desickling event. Apparently, therapy with potassium cyanate will be useful in the prophylaxis of sickle cell disease, but can be of no value in the management and early termination of acute sickle cell crisis.

Murayama (21, 22) demonstrated that intact homozygous S cells sickled by any of three physiologic methods (nitrogen, helium, or carbon dioxide) and subsequently desickled by urea (25 mmol/liter) in sugar solutions (equivalent to a **BUN** of 70 mg/100 ml) could again be sickled by adding urease and incubating at 37°C. Furthermore, Murayama noted that hemoglobin S erythrocytes, previously carbamylated with potassium cyanate by the method of Cerami and Manning, were not visibly affected by subsequent incubation with urease (21, 22).

Because of our clinical interest in verifying the therapeutic value of our recommended **BUN** concentrations in our published protocols (3–5) for intravenous therapy with urea–sugar solution in acute sickle cell crisis (requiring **BUN** concentration of 150–200 mg/100 ml), we repeated the urease experiments of Murayama with urea concentrations of 50 instead of 25 mmol/liter (see Methods). Otherwise, the conditions of our experiment were identical with those of Murayama. Figures 1–3 illustrate that urea in a 50 mmol/liter concentration (equivalent to a **BUN** concentration of about 140 mg/100 ml) in the presence of artificially induced sickling (by exposure to nitrogen in an oxygen-free atmosphere) inhibits sickling induced by physiologically comparable methods, and that destruction of the urea by urease causes reversion of erythrocytes to the sickled form. From this demonstration it follows that urea itself and not the cyanate ion is the molecule that affects desickling.

Clearly, the carbamylation of the beta S globin

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Table 2. Comparison of Effects of Urea and Potassium Cyanate on Sickling

<table>
<thead>
<tr>
<th>Urea</th>
<th>Potassium cyanate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverses and blocks sickling</td>
<td>Blocks but does not reverse sickling</td>
</tr>
<tr>
<td>Effects are reversible</td>
<td>Effects are irreversible</td>
</tr>
<tr>
<td>Sickled cells, reversed with urea, are again sickled after action of urease</td>
<td>Urea has no effect on cyanate-treated cells</td>
</tr>
<tr>
<td>Effects are immediate</td>
<td>Effects require time</td>
</tr>
<tr>
<td>Effects are apparent in the deoxygenated state</td>
<td>Initially hemoglobin S must be oxygenated before carbamylation will occur</td>
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CLINICAL CHEMISTRY, Vol. 18, No. 9, 1972 963
chain alters the quaternary structure or molecular conformation of the hemoglobin S molecule in such a way that steric hindrance prevents tetramer-tetramer interaction and, hence, the sickling of the hemoglobin S molecules. These findings with the carbamylation of hemoglobin S lend additional support for the modified Murayama hypothesis. The insight gained at the molecular level by the actions of urea and potassium cyanate on the sickling event support the modified Murayama hypothesis.

Both the urea and cyanate approaches are valid and independent attempts to use molecular information in an analytical and deductive manner for the development of effective chemotherapy for sickle cell disease. Both compounds act by separate and distinct molecular mechanisms to interfere with the sickling event. This conclusion above has been supported independently by Ranney (23) for other reasons.

The clinical implication of the information in this paper is that potassium cyanate may prove to be useful in the prophylactic management of sickle cell disease but it will be without value in treating and terminating acute sickle cell crisis.

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References

9. Murayama, M., Titratable sulfhydryl groups of normal and sickle cell hemoglobins at 0° and 38°C. J. Biol. Chem. 228, 231 (1957).
22. Murayama, M., Personal communication.

Editor's note: While this paper was in press, another paper bearing on this important question appeared: New Engl. J. Med. 287, 69 (also editorial, p 98) (1972).