Molecular and Cellular Effects of Antisickling Concentrations of Alkylureas

By Danek Elbaum, Eugene F. Roth, Jr., Gertrude Neumann, Ernst R. Jaffé, Robert M. Bookchin, and Ronald L. Nagel

Alkylureas are capable of inhibiting sickling in vitro and the gelation of solutions of hemoglobin S at concentrations between 0.05 and 0.1 M with increasing effectiveness that is directly proportional to the length of the alkyl chain (butyl > propyl > ethyl > methyl). The inhibitory effect is independent of pH between 6.5 and 7.5 and is a process driven by entropy. The alkylureas at concentrations of 0.1 M have minimal effects on several erythrocyte functions. Oxygen equilibria, osmotic fragility, reduced glutathione content, and glutathione reductase activity are totally unaffected, while pyruvic kinase activity is decreased only by butylurea by about 20%, and glucose-6-phosphate dehydrogenase activity is decreased progressively to a maximum of 30% in direct proportion to the length of the alkyl chain. Alkylureas not only inhibit sickling but are also capable of desickling erythrocytes that have been maintained in the deoxygenated state. They have little effect on several erythrocyte functions at antisickling concentrations, but their toxicity must be evaluated before they can be examined as potential therapeutic agents for the treatment or prevention of acute episodes in sickle cell anemia.

The polymerization of deoxy hemoglobin S is the underlying basis of red cell sickling and has attracted considerable scientific interest in the last few years. Allen and Wyman in 1954 and Murayama in 1957 observed that deoxy hemoglobin S gels with a negative temperature coefficient, suggesting that hydrophobic interactions were an important participant of the polymerization process. We have recently established that relatively low concentrations of alkylureas are capable of inhibiting the gelation of solutions of HbS, as well as inhibiting red cell sickling. The work presented here extends our knowledge of the basis and characteristic of the antisickling properties of alkylureas. In addition, we report their effect on some of the metabolic functions of red cells.

MATERIALS AND METHODS

Heparinized venous blood was obtained from persons homozygous for HbS (but whose red cells contained no more than 6% HbF) and from normal individuals. Hemolysates were prepared by the method of Drabkin with minor modifications. Solutions of hemoglobin were concentrated by vacuum ultrafiltration with simultaneous dialysis against 0.15 M potassium phosphate buffer, pH 7.35, at 4°C.

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Minimum Gelling Concentration Determinations

Minimum gelling concentrations (MGC) of deoxy HbS were determined as previously described\(^8\) at 22°-23°C, except as specified in the text. The concentration of hemoglobin determined spectrophotometrically after conversion to the cyanmet form using a molar extinction coefficient of 1.25 \(\times 10^4\) at 540 nm.

Potassium Loss

Measurement of potassium loss from cells was determined according to Segel et al.\(^9\) using an Instrumentation Laboratories flame photometer, Model 143.

Deoxygenation of red cell suspensions was accomplished using moist N\(_2\) gas containing 5.74\% (volume) CO\(_2\) at room temperature for 45 min. Deoxygenated red cell suspensions were centrifuged below films of mineral oil for 10 min at 250g.

Desickling of Red Cells

To study the capacity of alkylureas to unsickle already sickled cells, a double-well custom-made tonometer was utilized. In one well, an aliquot of whole blood from a HbS homozygous individual was placed. The other well contained 0.2 \(M\) ethylurea in 0.15 \(M\) phosphate buffer. Both wells were simultaneously deoxygenated by a stream of moist helium for 30 min at 23°C. The tonometer was gently shaken by hand. Upon completion of the deoxygenation, mixing of the ethylurea and red cells was accomplished by tilting the tonometer. Aliquots of deoxygenated blood, before and after the addition of ethylurea, were transferred anaerobically into an isotonic buffered 10\% formalin solution (pH 7.35). Red cell morphology was examined by two observers with a Zeiss microscope equipped with differential interference contrast optics. We classified as deformed any cell that was not a biconcave disc. The designation of sickled cells was reserved for cells with at least one sharp or pointed aspect at the perimeter.

Osmotic Fragility

Osmotic fragility was determined according to the method of Dacie and Lewis,\(^10\) except that in the experimental samples, one of the alkylureas (methyl-, ethyl-, propyl-, or butylurea) was present in the buffered saline at a final concentration of 0.1 \(M\).

Red Cell Enzymes

The assays of glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), and pyruvate kinase (PK) activity were performed according to the method of Beutler,\(^11\) except that in the experimental samples the cuvettes containing cell lysate, substrate, cofactors, and buffer also contained the indicated alkylurea at a final concentration of 0.1 \(M\).

Methemoglobin Reduction

This red cell function was assayed as described previously,\(^12\) except that in the experimental samples the red cells were incubated with the indicated alkylurea at a final concentration of 0.1 \(M\). Glucose utilization was determined as described previously,\(^12\) Heinz body induction tests in normal red cells with acetylphenylhydrazine were performed as described by Dacie and Lewis.\(^10\) Reduced glutathione was determined according to Beutler.\(^11\)

Red Cell ATP

Heparinized blood was drawn from normal volunteers, the erythrocytes washed three times with isotonic sodium chloride solution, and the buffy coat discarded. The cells were suspended in glycyl-glycine buffer (pH 7.4) with 10 mM glucose as described previously,\(^12\) at a packed cell volume of 25\%. Alkylureas, dissolved in the same buffer, were added in final concentrations up to 0.5 \(M\). The cell suspensions were incubated at 37°C in a shaking water bath for 24 hr. At the end of this period, aliquots were precipitated with equal volumes of 12\% cold trichloroacetic acid for determination of red cell ATP, using the UV Sigma Kit procedure (Sigma Chemical Co., St. Louis, Mo.). Hemoglobin concentration was measured by the cyanmethemoglobin method, and results were expressed as micromoles ATP/g hemoglobin.
Scanning Electron Microscopy

Red cells were fixed in buffered isotonic 5% formalin and then dehydrated in a series of alcohols of increasing concentration. Samples were transferred to Freon I1 I and then subjected to critical point drying. Samples were sputtered with gold and viewed on an ISI Super MiniSEM.

RESULTS

The effects of ethylurea and propylurea on minimum gelling concentrations (MGC) are illustrated on Fig. 1. Increasing concentrations of both ethylurea and propylurea result in a progressive inhibition of gelation. Propylurea is a more potent inhibitor than ethylurea in the concentration range from 0.05 to 0.4M.

Table 1 shows the effect on MGC of propylurea and compounds that have the same number of carbons but correspond to different configurations or chemical functions. Propylurea is the most effective. Compounds with branched alkyl chains or with an NH group eliminated (amides), although still inhibitory, are less effective than the prototype compounds. Citrulline is totally ineffective.

Table 1. Effect of 0.1 M Urea Derivatives on MGC of HbS

<table>
<thead>
<tr>
<th>Urea Derivative</th>
<th>MGC (g/100 ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>24.0</td>
</tr>
<tr>
<td>(\text{NH}_2\text{C}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_3) (propylurea)</td>
<td>33.4</td>
</tr>
<tr>
<td>(\text{NH}_2\text{C}-\text{NH}-\text{CH}_3) (isopropylurea)</td>
<td>31.8</td>
</tr>
<tr>
<td>(\text{NH}_2\text{C}-\text{CH}_2\text{CH}_2\text{CH}_3) (butyramide)</td>
<td>31.2</td>
</tr>
<tr>
<td>(\text{NH}_2\text{C}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}-\text{COO}) (citrulline)</td>
<td>24.39</td>
</tr>
</tbody>
</table>
The effects of pH variations on the MGC of HbS solutions in the presence and absence of ethylurea are shown in Fig. 2. At all pHs studied, the effect of ethylurea is equivalent. In other words, the inhibiting effect of ethylurea on HbS gelling is not pH-dependent in the range between 6.6 and 7.4.

The effect of temperature on the gelling of HbS in the presence or absence of ethylurea is shown in Fig. 3. As can be observed, the slope of both curves is equivalent. The calculation of $\Delta H$ in both cases shows that the enthalpy in the presence or absence of ethylurea is about 1500 calories/mole. This finding strongly suggests that the inhibition of gelling is an entropic effect.

Measurements of the osmotic fragility of normal and HbS-containing red cells in the presence of 0.1 M methyl-, ethyl-, propyl-, and butylurea are presented in Figs. 4A and B. The osmotic fragility of either normal or HbSS-containing cells is unaffected by the presence of the alkylureas in these concentrations.

The effects of 0.1 M ethylurea on the morphology of previously sickled and normal red cells at two different temperatures (37°C and 4°C) are summarized in Fig. 5. At 37°C, 0.1 M ethylurea can desickle approximately 50% of previously sickled cells. This effect almost completely disappears at 4°C. No effect of the same concentration of ethylurea is observed on normal red cell morphology.

The loss of potassium from sickled cells to the medium was studied as a function of time. Several authors have demonstrated that K⁺ loss was associated
with the sickling of HbS-containing cells. Figure 6 shows that 0.1 M ethylurea reduced potassium loss from deoxygenated (less than 5% oxyhemoglobin) sickled cells. Oxy normal red cells incubated with 0.1 M ethylurea had a K\(^+\) leak indistinguishable from oxy sickle cells (not shown in Fig. 6).

The ability of red cells to reduce nitrite-induced methemoglobin in the presence of 0.1 M alkylureas is shown in Fig. 7. The cells were maintained at 37\(^\circ\)C and observed over a 22\(\frac{1}{2}\) hr period. When glucose was omitted from the

![Figures](https://via.placeholder.com/150)

**Fig. 4.** The effect of alkylureas on the osmotic fragility of normal (A) and SS (B) red cells at 23\(^\circ\)C. e, control; ⃝, 0.1 M methylurea; ⋁, 0.1 M ethylurea; ×, 0.1 M propylurea; ⧫, 0.1 M butylurea.

**Fig. 5.** Effect of 0.1 M ethylurea on the morphology of previously sickled and normal cells at 37\(^\circ\)C. Ethylurea was dissolved in 0.15 M potassium phosphate buffer (pH 7.35). Ordinate, S + D, represents the sum of sickle + deformed cells expressed as a percentage of the total cells. The fraction of sickle (solid) and deformed (slanted lines) is depicted in each bar.
incubation medium, no methemoglobin reduction took place, indicating that methemoglobin reduction was an energy-requiring process. None of the alkylureas had any discernible effect on this process in the presence of glucose. When this experiment was repeated with HbSS red cells, the same results were obtained.

None of the alkylureas studied had any effect on glucose utilization during this same time period as compared with the control. After 5 hr, about 80% of the initial glucose was present. At 22 hr, about 55% of the glucose remained. Similarly, glucose utilization followed in sickle cells incubated with ethylurea was indistinguishable from that in the control.

The effects of alkylureas (0.1 M) on the activities of three red cell enzymes are shown in Fig. 8. There was no significant effect on the activity of glutathione reductase. G6PD and, to a lesser extent, pyruvate kinase were slightly inhibited by the longer alkylureas, propyl- and butylurea. The inhibition appeared to increase according to the length of the alkyl group. However, the maximum inhibition was only about 20%–25% of the control values. These enzyme assays were repeated with erythrocytes from individuals with sickle cell anemia with identical results.

The GSH content of normal (or sickle) cells treated with alkylureas at a
Fig. 8. The effect of 0.1 M alkylureas on the activities of glutathione reductase, pyruvate kinase, and glucose-6-phosphate dehydrogenase. GR, PK; G-6-PD. The bars show the range of results. Ordinate: per cent activity with 100% corresponding to the control.

0.1 M concentration was not significantly reduced (Fig. 9). No increase in Heinz body formation was found in the presence of alkylureas.

The effects of 0.1 M methyl-, ethyl-, propyl-, and butylurea on red cell ATP are shown in Table 2. The values at zero time are slightly low, and reflect washing and handling procedures. At 24 hr, the ATP content has returned to the normal range with no discernible difference between control and alkylurea samples. The effects of ethylurea at concentrations up to 0.5 M for 24 hr are shown in Table 2. Once again, no significant differences are seen, and all results are in the normal range. The samples containing 0.4 and 0.5 M ethylurea show slight hemolysis after 24 hr.

Scanning electron microscopy showed no differences between red cells incubated in 0.1 M butylurea and normal controls (Fig. 10).

DISCUSSION

The present studies extend our understanding of the mechanism of inhibition of sickling and gelation by the alkylureas. We have shown previously that both gelation of deoxy HbS and erythrocyte sickling are inhibited by alkylureas, to an extent proportional to the length of the alkyl chain.6 These inhibitory effects, as well as the actions of higher concentrations of alkylureas in promoting the denaturation13 or subunit dissociation14 of hemoglobin, are thought to result from reversible interference with hydrophobic interactions.
Table 2. Effect of Alkylureas on Normal Red Cell ATP

<table>
<thead>
<tr>
<th></th>
<th>ATP (µ moles/g Hb)</th>
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<tbody>
<tr>
<td></td>
<td>0 Time</td>
</tr>
<tr>
<td>Control</td>
<td>3.00</td>
</tr>
<tr>
<td>Methylurea 0.1 M</td>
<td>2.95</td>
</tr>
<tr>
<td>Ethylurea 0.1 M</td>
<td>2.85</td>
</tr>
<tr>
<td>Propylurea 0.1 M</td>
<td>2.95</td>
</tr>
<tr>
<td>Butylurea 0.1 M</td>
<td>2.66</td>
</tr>
<tr>
<td>Control</td>
<td>3.73</td>
</tr>
<tr>
<td>Ethylurea 0.2 M</td>
<td>3.71</td>
</tr>
<tr>
<td>Ethylurea 0.3 M</td>
<td>3.71</td>
</tr>
<tr>
<td>Ethylurea 0.4 M</td>
<td>3.67</td>
</tr>
<tr>
<td>Ethylurea 0.5 M</td>
<td>3.70</td>
</tr>
</tbody>
</table>

The finding that the inhibition of gelling is independent of pH over the range of about 6.5–7.5, the region over which α-amino groups, sulfhydryl groups, histidine, and certain lysine groups ionize, suggests that the inhibitory effects of alkylureas are not mediated by altered interactions of these groups.

The small magnitude of enthalpy change (ΔH) measured for the gelation process (about 1.6 Kcal/mole)* is consistent with the findings and conclusions of Ross, Hofrichter, and Eaton3 that gelation of deoxy HbS is an entropically driven process. Furthermore, since ΔH is found to be unchanged upon partial inhibition of gelling by alkylureas, the inhibitory action per se must also be entropically driven.

Comparison of a group of analogous compounds (Table 1) indicates that the straight chain compound, propylurea, has a more effective conformation for inhibiting gelation of deoxy HbS than the branched alkylurea or the corre-

*Note that this figure represents the general order of magnitude but is not a precise determination of enthalpy, since no corrections have been made for nonideality.
sponding amide, while citrulline, an analogue having a relatively polar chain end, is totally ineffective.

The gelation-inhibitory effect of increasing concentrations of propyturea is consistently greater than equivalent concentrations of ethylurea in the range tested (Fig. 1). The decreasing slope of rise in MGC with increasing concentrations of both alkylureas probably reflects the increased protein–protein and protein–solvent interactions (nonideality) at these very high protein concentrations.

Turning now to the effects of alkylureas on intact erythrocytes, we see that in addition to ethylurea’s ability to inhibit sickling as reported previously, it is capable of reversing the sickling of deoxygenated, sickled SS red cells (Fig. 5). Thus it is both an antisickling and a desickling agent.

The extent of increase in the rate of K+ loss from SS red cells upon deoxygenation-induced sickling has been used as a quantitative assessment of sickling. The finding that addition of 0.1 M ethylurea slowed the rate of K+ loss from deoxygenated SS red cells at 37°C, but did not reduce the rate to that of normal (HbA) cells, was consistent with the partial inhibition of sickling observed under these conditions. Furthermore, the lack of effect of 0.1 M alkylureas on the osmotic fragilities of normal and SS red cells suggested that they produce no dramatic deleterious effects on membrane permeability.

The major pathway for reduction of methemoglobin in red cells appears to require an NADH-dependent methemoglobin reductase, and thus an intact Embden–Meyerhof glycolytic pathway. The normal rate of methemoglobin reduction and normal glucose utilization in the presence of 0.1 M alkylureas suggest that none of the steps of this pathway is significantly disrupted.

G6PD is the first enzyme of the hexose monophosphate shunt pathway whereby NADPH is generated for reduction of oxidized glutathione (GSSG to GSH). Normally, only 10% of red cell glucose metabolism proceeds via this pathway, although the shunt can be stimulated to augment this proportion. The observed degree of inhibition of G6PD by propyl- or butylurea is not likely to result in hemolysis such as may occur in X-linked G6PD deficiency during infection or drug-induced oxidative stress. This conclusion is supported by our findings that alkylureas do not increase Heinz body formation in red cells incubated with acetylphenyl-hydrazine, and produce no significant decrease in the levels of GSH. On the other hand, these longer chain alkylureas might have clinical consequences in persons with hereditary G6PD deficiency. Other red cell enzyme and metabolic functions tested are either minimally affected (e.g., pyruvate kinase activity in the presence of butylurea) or unaffected (glutathione reductase activity, red cell ATP levels).

We have previously shown that the intrinsic oxygen equilibria of hemoglobin solutions and whole blood are unaltered by alkylureas. The present findings show that the antisickling and desickling actions of these agents (which presumably act in a noncovalent manner) occur without disruption of other principal functions of the red cells.

It is not yet possible to predict whether or not alkylureas will be useful preventative or therapeutic agents in sickle cell disease, but the in vitro data we
have accumulated suggest that toxicologic studies are warranted as the next step in assessing their clinical potential.

ACKNOWLEDGMENT

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REFERENCES

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