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Effect of Alkylureas on the Polymerization of Hemoglobin S

(protein chemistry/sickling/gelation/hydrophobic interactions/sickle cell anemia)

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ABSTRACT **Alkylureas (methyl-, ethyl-, propyl-, and butyl-) can inhibit both the gelation of deoxyhemoglobin S and red cell sickling without denaturation of the hemoglobin or intrinsic alteration of its oxygen affinity. This effect is directly proportional to the length of the alkyl chain and substantiates the importance of hydrophobic interactions in the polymerization of hemoglobin S. In addition, it opens the possibility that further systematic investigations with these compounds will help quantitate the role of hydrophobic interactions in this system so as to further our understanding of the polymerization of deoxyhemoglobin S.**

The mutation of hemoglobin S (Hb S), which consists of replacement of a polar residue (glutamic acid) by a hydrophobic residue (valine) in position 6 of the β chains (1), renders the molecule capable of polymerizing in the deoxy form, with the formation of tactoids (2). Intracellular ordered aggregates have been demonstrated in deoxygenated erythrocytes from persons with sickle cell anemia and studied by electron microscopy (3–6), by measurements of low-angle x-ray diffraction (7), linear birefringence (8), and dichroism (6, 9). The polymers, elongated microtubules having diameters of about 180 Å, deform the erythrocyte (sickling) and alter their rheological properties. The *in vitro* correlate of the linear aggregation in red cells is the capacity of a solution of deoxy-Hb S to gel. This is a concentration-dependent process, which probably occurs in a concerted manner (10).

Allen and Wyman (11) and Murayama (12) observed that deoxy-Hb S gels with a negative temperature coefficient. Although initial reports concerning the anti-sickling effects of 0.1–0.2 M solution of urea (13) could not be confirmed, Cerami and Manning (14) have observed that rather high concentrations of urea (1 M) are capable of interfering with gelation and sickling. This has been confirmed by Segal *et al.* (15). Although these findings suggest that hydrophobic interactions are involved in the polymerization of deoxy-Hb S, detailed studies of such interactions are lacking.

The capacity of low concentrations of alkylureas to promote the dissociation of hemoglobin from tetramers to dimers without denaturation, and the observed correlations between the degree of such dissociation and the relative hydrophobicity of a series of alkylurea compounds (16), prompted us to investigate the effect of these compounds on gelation of deoxy-Hb S and on red cell sickling. The results of the initial studies described in the present report demonstrate the potential of these agents in furthering our understanding of the mechanism of deoxy-Hb S and of hydrophobic interactions in general.

Abbreviation: Hb, hemoglobin.

MATERIALS AND METHODS

Heparinized venous blood was obtained from patients homozygous for Hb S (*SS*) (who have sickle cell anemia) and from normal individuals, and hemolysates were prepared by the method of Drabkin (17) with minor modifications. Solutions of hemoglobin were concentrated to 30–36 g/dl by vacuum ultrafiltration at 4° with simultaneous dialysis against 0.15 M potassium phosphate buffer (pH 7.35). Minimum gelling concentrations of deoxy-Hb S were determined at 22–23° by the method of Singer and Singer (18), as modified by Bookchin *et al.* (19).

Oxygen equilibria of dilute solutions of hemoglobin (about 12.5 μM tetramer) were determined at 22° by the methods of Allen *et al.* (20) and Riggs (21). Solutions were deoxygenated with helium gas in tonometers with integral cuvettes [by the criterion of Benesch *et al.* (22) for complete deoxygenation, a ratio of 1.23–1.25 for A_{555}/A_{540}]. Air was then added to the tonometers in stepwise increments with a calibrated pipette, as described by Rossi-Fanelli and Antonini (23), and the degree of oxygen saturation was determined spectrophotometrically with a Cary 14 recording spectrophotometer. Oxygen equilibria of whole blood samples were measured at 37° with a model 182 CO-oximeter, IL 213 pH/Blood Gas Analyzer, and IL 237 tonometer (Instrumentation Lab. Inc.).

The oxygen equilibrium of hemoglobin can be represented as follows:

$$Hb_4 + nO_2 \rightleftarrows Hb_4(O_2)_n$$

where one binding constant K_B is defined by the following equation:

$$K_B = \frac{Hb_4(O_2)_n}{(Hb_4)\ (O_2)^n} \qquad [1]$$

Taking logarithms of both sides of Eq. [1], the Hill equation (24) can be derived.

$$\log \frac{S}{1-S} = \log K_B + n \log pO_2 \qquad [2]$$

where S represents the fraction of saturation with oxygen, pO_2 is the partial pressure of oxygen (mm of Hg), and n is the apparent order of the reaction or the Hill coefficient. The oxygen equilibrium data have been plotted with Eq. [2].

Hemoglobin concentrations were determined spectrophotometrically after conversion to the cyanmet form with Drabkin's reagent, using the molar extinction coefficient of 1.25×10^4 at 540 nm. Measurements of optical rotatory dispersion were made on a Cary 60 recording spectropolarimeter, and were

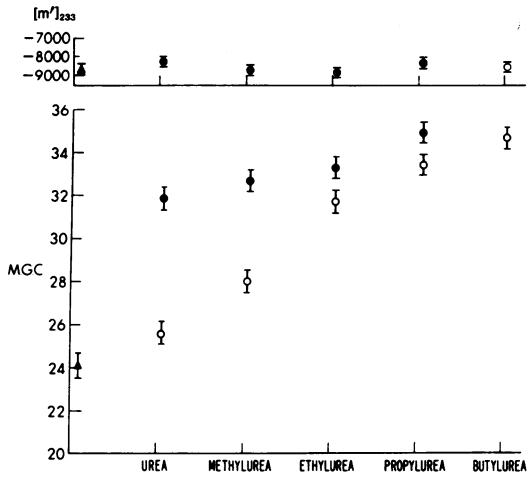

FIG. 1. The effects of alkylureas on the minimum gelling concentration (MGC); in g/100 ml and mean residue rotation at 233 nm ($[m']_{233}$) of Hb S. ▲, Control with no alkylurea; O, 0.1 M alkylureas; ●, 0.2 M alkylureas. All solutions contained 0.15 M potassium phosphate buffer (pH 7.35). Temperature, 23°. Ranges of repeated observations are shown.

calculated according to the following equation:

$$[m']_{233} = \frac{\alpha M_0}{cl} \cdot \frac{3}{(n^2 + 2)}$$

where α is the observed rotation at wavelength 233 nm, c is the concentration of protein in g/dl, n is the refractive index of the solvent at this wavelength, and l is the path length in decimeters. The mean residue molecular weight M_0 of 112.5 was used.

As a measure of red cell deformability, suspensions of red cells in isotonic phosphate-buffered saline (pH 7.2) containing 0.25% bovine serum albumin were filtered through polycarbonate filters having pore diameters of 3 μm (Nucleopore, lot no. 2373) by the methods of Fleischer *et al.* (25) and Gregersen *et al.* (26). The filters were mounted on a stainless steel grid clamped in a filter funnel (Millipore Corp.), and 0.1 ml of red cell suspensions with hemoglobin concentrations of 2 g/dl were filtered at room temperature both in the presence and in the absence of oxygen. For filtration in the deoxy state, about 10 ml of red cell suspension were first placed in a 200-ml tonometer and equilibrated at 37° with 5% CO_2 in N_2 gas saturated with water. The level of oxygen saturation and hemoglobin concentration were monitored with an IL model 182 CO-oximeter. Since determination of the rates of filtration of the red cell suspensions were found to be poorly reproducible, the maximum number of filtered cells was measured, with a Coulter Counter model F (Coulter Electronics, Inc.).

In order to study the effects of alkylureas on red cell sickling, 6 ml of whole blood was equilibrated in a tonometer with 5.6% CO_2 in air for 15 min, after which baseline measurements of pH, O_2-Hb, and CO-Hb were made. Deoxygenation was then performed in six to eight steps (the final step being 5.6% CO_2 in N_2), each with a 15-min gas equilibration, followed by the above measurements. At each step, and also after a 15-min reoxygenation, an aliquot of blood was transferred anaerobically into 10% formalin in isotonic phosphate-buffered saline (pH 7.35). Red cell morphology was examined with a Zeiss microscope equipped with differential interference contrast optics.

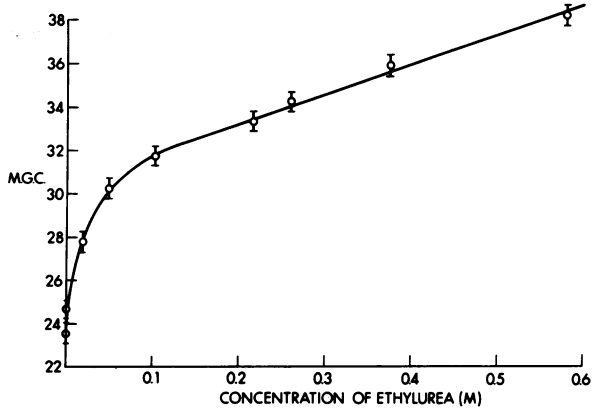

FIG. 2. The effect of increasing concentrations of ethylurea solution on the minimum gelling concentration (MGC; in g/100 ml) of deoxy Hb S. Temperature, 23°. Ethylurea was dissolved in 0.15 M potassium phosphate buffer (pH 7.35). Ranges of repeated observations are shown.

RESULTS

The effects of equivalent concentrations of various alkylureas (0.1 M and 0.2 M) on the gelation of deoxy-HbS are illustrated in Fig. 1. Each of the five agents tested was found to inhibit gelation to a degree that was approximately proportional to the number of methylene units or to the length of the hydrocarbon alkyl group of the urea. Thus, 0.1 M urea produced a very small increase in the minimum gelling concentration (to 26 g of Hb/dl from a control of 24), whereas the greatest inhibition occurred in the presence of 0.1 M butylurea, which raised the minimum gelling concentration to 34 g of Hb/dl. In Fig. 2, it is shown that increasing concentrations of ethylurea result in progressive inhibition of gelation. A concentration of 0.3 M ethylurea was required to raise the minimum gelling concentration to the same extent as did 0.1 M butylurea.

Optical rotatory dispersion measurements showed no significant alterations in the α-helical content of oxy-Hb S in the presence of 0.2 M alkylureas. The mean residue rotations of the solutions at 233 nm varied between −8400 and −8900, as compared with −8700 ± 300 in the absence of the ureas. Thus, the variations seen in the presence of the ureas (which correspond to a 4% variation in the α-helical content of the protein) are within the experimental error of the method.

The oxygen equilibria of whole normal and sickle cell blood, and of solutions of Hb A, in the presence of 0.1 M alkylureas are shown in Figs. 3 and 4. Addition of 0.1 M ethylurea produces no change in the oxygen affinity or cooperativity of normal red cells (p50 = 29 mm of Hg, n = 2.7) (Fig. 3A), but in sickle cell blood (Fig. 3B), results in a slight increase in oxygen affinity (p50 decreases from 33 to 31 mm of Hg) and a small decrease in the Hill coefficient (n = 2.3 as compared with 2.8 in the control of the same blood sample).

Dilute solutions of Hb A showed no alterations of oxygen affinity or cooperativity in the presence of 0.1 M ethylurea or propylurea (Fig. 4) (p50 = 8 mm of Hg, n = 2.7). With 0.1 M butylurea, there was a small increase in oxygen affinity (p50 = 7 mm of Hg) but cooperativity remained normal.

With much higher concentrations of alkylureas, there were

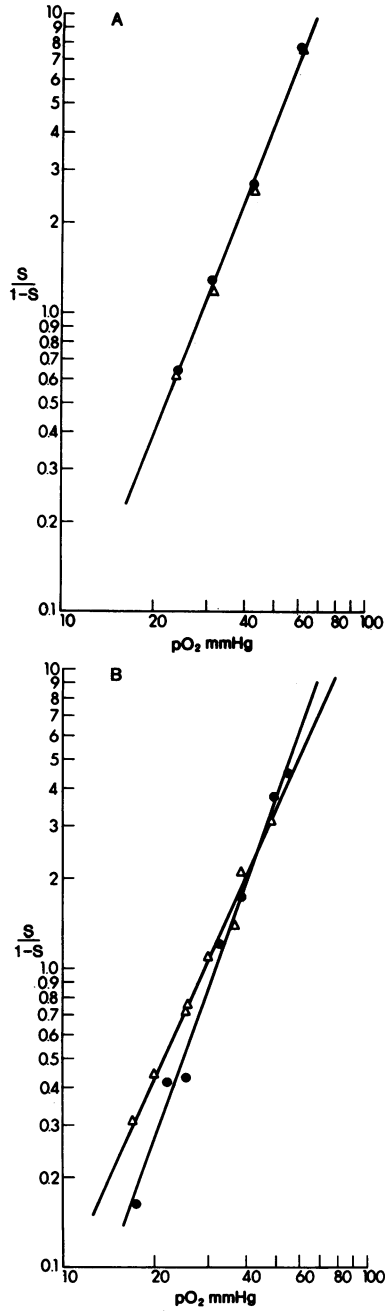

FIG. 3. The oxygen equilibria of normal (A) and sickle cell (B) blood in the presence of 0.1 M ethylurea plotted according to Hill's equation (ordinate, fraction of oxygen saturation; abscissa, partial pressure of oxygen in mm of mercury). ●, Whole blood containing 0.1 M potassium phosphate buffer (pH 7.35) (control); △, whole blood containing 0.1 M ethylurea dissolved in 0.15 M potassium phosphate buffer (pH 7.35). Temperature, 37°.

differences in stability between hemoglobins A and S. Oxy-Hb S was found to precipitate in the presence of 1 M propylurea, whereas oxy-Hb A remained in solution and showed a normal α-helical content of about 70%.

When blood from persons with sickle cell anemia is gradually deoxygenated, the proportion of sickled cells varies with the percentage of oxy-Hb. Fig. 5 demonstrates the generation of sickled cells upon stepwise deoxygenation of whole sickle

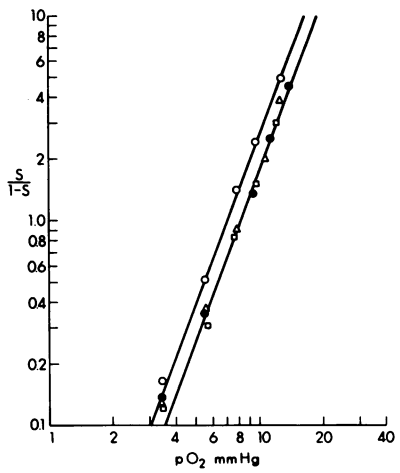

FIG. 4. The oxygen equilibria of hemoglobin A in the presence of 0.1 M alkylureas plotted according to Hill's equation (ordinate, fraction of oxygen saturation; abscissa, partial pressure of oxygen in mm of mercury). △, 0.1 M ethylurea; □, 0.1 M propylurea; ○, 0.1 M butylurea; ●, control. All solutions contained 0.15 M potassium phosphate buffer (pH 7.35). Concentration of hemoglobin, 16 mM. Temperature, 22.5–23°.

cell blood, in the presence of various alkylureas. In the control, newly sickled and deformed cells begin to appear when the average level of oxy-Hb falls below 60%, and upon complete deoxygenation, over 90% of the cells are deformed or sickled. The addition of alkylureas to a concentration in the blood of 0.1 M results in an inhibition of sickling throughout the range of deoxygenation. As seen in the gelation experiments, the extent of this inhibiting effect varies directly in proportion to the length of the hydrophobic portion of the alkylurea molecule: fully deoxygenated blood containing 0.1 M methyl-, ethyl-, and butylureas contained 87%, 77%, and 43% sickled plus deformed cells, respectively. Urea (0.1 M) in turn, did not exhibit any inhibitory effect on sickling and was indistinguishable from the control.

The effect of 0.1 M ethylurea on the filterability of normal

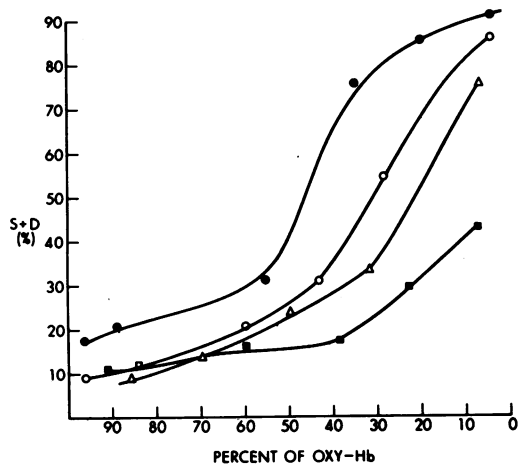

FIG. 5. The effect of alkylureas on the generation of sickled and deformed red cells by progressive deoxygenation. ○, 0.1 M methylurea; △, 0.1 M ethylurea; ■, 0.1 M butylurea; ●, control. All solutions contained 0.15 M potassium phosphate buffer (pH 7.35). S represents percent of sickled cells; D represents percent of deformed cells. Temperature, 37°.

TABLE 1. *Red cell filterability*

	Degree of oxygenation*	Filtration recovery (%)
Normal red cells (control)	Oxygenated	82 ± 10
	Deoxygenated	84
Normal red cells (0.1 M ethylurea)	Oxygenated	88
	Deoxygenated	76
Sickled red cells (control)	Oxygenated	84
	Deoxygenated	35
Sickled red cells (0.1 M ethylurea)	Oxygenated	90
	Deoxygenated	77

* Oxygenation means oxyhemoglobin no less than 94%; deoxygenation means oxyhemoglobin no more than 3%.

and of homozygous red cells for Hb S (*SS*) are shown in Table 1. Under the conditions described, the filterability of normal erythrocytes was not altered by addition of ethylurea, and no significant differences were found between oxygenated and deoxygenated red cell suspensions (over 80% recovery). The well-known decrease in filterability of deoxygenated *SS* red cells (27) was found to be significantly reversed in the presence of 0.1 M ethylurea: filtration recovery was elevated from 35% to 77%. As expected with the relatively low pressure gradients used in these experiments, the hemolysis index, I_{Hb}, as measured by Chien *et al.* (28), was less than 0.05, indicating no significant degree of hemolysis.

DISCUSSION

The most significant finding of the present investigation is the increasing order of effectiveness of alkylureas on the inhibition of the gelling of Hb S (Fig. 1) and red cell sickling (Fig. 5) in direct relationship with the length of the hydrophobic portion of the molecule.

It is of interest to note that the order of effectiveness of the alkylureas used in this study is the same as the order of effectiveness of these compounds as subunit dissociating agents of human hemoglobin (16) and as denaturing agents (29), studied at much higher alkylurea concentrations. The predictability of the effects of these compounds on the state of the quaternary structures of hemoglobin in solution opens the possibility of systematic investigations concerning the relationship between their structure and mode of action.

At the concentrations of alkylureas used in the experiments reported here, there was no evidence of dissociation or denaturation of the protein. Specifically, the oxygen equilibria and the α-helical content of the solutions of normal Hb exposed to these low concentrations of alkylureas were not altered. With Hb S, on the other hand, an abnormally low oxygen affinity is found, which appears to be related directly to the polymerization of the deoxy-Hb (30). It is therefore not surprising that the inhibition of sickling by the alkylureas partially corrects this right shift in the oxygen dissociation curve of red cells containing Hb S. A heterogeneous response of the molecular contents of the cells to the depolymerizing action of the alkylureas may account for the observed apparent decrease in the Hill coefficient (n-value).

The observed effects of the alkylureas on sickling and Hb S gelation supports the notion first suggested by Murayama (31) that hydrophobic forces contribute to the stability of Hb S polymers. It is the substitution of the nonpolar valyl side chain for the polar glutamic acid group at the surface of the Hb S molecule that results in the polymerization that underlies the sickling of red cells containing Hb S. Furthermore, it appears that the valyl side chain at this site [A3(6)β] is rather specific, for the occurrence of an alanine residue at the same site does not facilitate aggregation of the deoxy-Hb (32, 33).

In addition to hydrophobic interactions, it has become increasingly clear that the aggregation of deoxy-Hb S involves other weak intermolecular forces. Evidence presented by Bookchin and Nagel (34) and confirmed by Freedman *et al.* (35) and Briehl and Ewert (36) indicates that polar interactions between the molecules also play an important role.

While the effects of the alkylureas in solutions of deoxy-Hb S are clearly related only to their effects on polymerization and gelation, the effects on red cell sickling, deformation, and filterability might also involve alterations of the red cell membranes. The findings that alkylureas produce a decrease in the number of "irreversibly" sickled cells in the presence of oxygen suggests a separate action by which the rigidity and nondeformability of these membranes may be reversed.

No hastily derived conclusions can be drawn as to the applicability of the present findings to the management of sickle cell anemia. A thorough investigation of the potential toxicity of these compounds must be completed before any therapeutic application of the alkylureas can be considered.

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