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Potassium Cyanate as an Inhibitor of the Sickling of Erythrocytes *In Vitro*

(urea/hemoglobin S/carbamylated valine)

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ABSTRACT **The recent use of urea as a treatment for the crisis phase of sickle-cell anemia has prompted us to investigate the possibility that cyanate, which is in equilibrium with urea in solution, might itself prevent the sickling of erythrocytes. We have found that in contrast to the high concentration of urea (1 M) needed to prevent *reversibly* the *in vitro* sickling of 80% of the cells, potassium cyanate (0.01–0.10 M) *irreversibly* inhibits sickling to the same extent. The prevention of sickling is a function of the amount of [^{14}C]cyanate incorporated into acid-precipitable protein (0.1–1.0 mol of cyanate per mol of hemoglobin). Most of the radioactivity is accounted for by carbamylation of the NH_2-terminal valine residues of hemoglobin; there is no detectable carbamylation of the lysine or cysteine residues. The reactive species, HN=C=O (isocyanic acid), may be an analog of O=C=O since both compounds bind to the same valine residues of hemoglobin. Deoxygenated sickled cells also incorporate [^{14}C]-cyanate, but the sickling is not reversed. Oxygenation results in normal morphology in 75% of these cells. Upon subsequent deoxygenation, these cells remain normal. Potassium cyanate (5 mM) was also found to be an effective inhibitor of the gelling of deoxyhemoglobin S.**

Recently, successful treatment of the crisis phase of sickle-cell anemia by the administration of large amounts of urea in a solution of invert sugar has been reported (1–3). The rationale (4) for this therapy was that the urea presumably disrupted the hydrophobic bonds supposedly formed between sickle hemoglobin molecules, a hypothesis based upon observations by Muryama (5) and Perutz and Lehmann (6). Although urea is a well-known protein denaturant, concentrations of 1–8 molar are usually required for the unfolding of a protein (7). Hence, it seemed unlikely that the reported long-term clinical improvement of the patients was due to the urea, since urea is rapidly cleared from the blood. We have, therefore, investigated the possibility that cyanate, which is in equilibrium with urea in solution (8) (Eq. 1), might itself prevent sickling.

$$NH_2-\overset{\overset{O}{\|}}{C}-NH_2 \rightleftharpoons NH_4^+ + {}^-NCO \quad (1)$$

The cyanate, which is present in urea solutions, has been shown by Stark, Stein, and Moore (9) to carbamylate the ϵ-NH_2 groups of lysine residues and the NH_2-terminal residues of proteins (Eq. 2). In this communication, we show that potassium cyanate reacts with erythrocytes from patients with sickle-cell anemia to prevent sickling, and also reacts with isolated hemoglobin S (HbS, the hemoglobin from sickle-cell erythrocytes) to prevent gel formation.

$$R-NH_2 + HN=C=O \longrightarrow R-\overset{\overset{H}{|}}{N}-\overset{\overset{O}{\|}}{C}-NH_2 \quad (2)$$

MATERIALS

The Ultrapure grade of urea was obtained from Mann; KCNO was purchased from Baker and Adams; K^{14}CNO was obtained from New England Nuclear Corp. Blood from sickle-cell anemia patients was drawn into tubes containing either heparin or EDTA. The erythrocytes were washed three times with Dulbecco's phosphate-buffered saline (PBS), pH 7.40 (10) and suspended in PBS to a final hemoglobin concentration of 130 mg per ml (2 μmol per ml). The experiments were always conducted within 24 hr after the blood was drawn. In order to ensure complete oxygenation, the suspension of cells was bubbled gently with air for a few minutes.

METHODS

Assay for *in vitro* sickling

The morphology of sickle-cell erythrocytes depends on the extent of saturation of HbS with oxygen (11). When fully oxygenated, most sickle-cell erythrocytes have normal morphology (see Fig. 1*a*); upon deoxygenation these cells sickle. In the present study, we have used the following procedure to determine the amount of sickling. The cell suspension was diluted ten-fold with PBS; the solution was deoxygenated by evacuation* at 30 mm of Hg with a water aspirator for 7 min at 37°C. The evacuated tubes were then incubated an additional 5 min at 37°C and the cells were fixed by rapid dilution with buffered formalin. These conditions of deoxygenation regularly produced abnormal morphology in 80–90% of the cells (see Fig. 1*b*). The cells were counted by two different observers using a Wild phase-microscope (×600).

Incorporation of [^{14}C]cyanate into erythrocyte protein

Sickle-cell erythrocytes were incubated with [^{14}C]cyanate, and the amount of radioactivity incorporated into protein was determined after precipitation of the cells with cold 5% trichloroacetic acid (TCA). The precipitate was collected on a 0.45-μm Millipore filter and washed four times with 10 ml of

Abbreviations: HbS, hemoglobin S; TCA, trichloroacetic acid; PBS, phosphate-buffered saline (10).

* We have repeated many of the experiments described in this paper using venous gas pressure (3.81% O_2, 5.61% CO_2, 90.58% N_2) as the means to induce sickling and have obtained similar results.

TABLE 1. *Effect of urea and KCNO on sickling of deoxygenated erythrocytes**

Expt.	Addition	Concentration during incubation (M)	Concentration during deoxygenation (M)	Normal deoxygenated cells (%)
1	—	—	—	17
2	Urea	1.0	1.0	69
3	Urea	—†	1.0	70
4	Urea	1.0	0.1	21
5	Urea	0.1	0.1	22
6	KCNO	0.1	0.01	72
7	KCNO	0.01	0.001	34

Oxygenated erythrocytes (2 μmol HbS/ml) were incubated for 1 hr at 37°C. The cells were then diluted into the appropriate buffer and deoxygenated as described in *Methods*.

* An oxygenated sample had 93% normal cells.

† The cells in Expt. 3 were deoxygenated immediately.

5% TCA. The filter and the protein were placed in a sample solubilizer (NCS, Amersham/Searle) and the samples in scintillation fluid were counted in a Packard scintillation counter Model 3380.

The site of carbamylation in the erythrocyte was determined as follows: the cells (about 50 mg of Hb) that had been incubated with cyanate were precipitated and washed four times with 5 ml of cold 5% TCA. After removal of the TCA by extraction with ether, the protein was dried at 40°C and then was dissolved in 1–2 ml of 50% acetic acid. A portion of this solution was heated in 6 N HCl at 110°C for 22 hr (12); amino acid analysis of the hydrolysate was done as described by Spackman, Stein, and Moore (13). The amount of homocitrulline present in this hydrolysate is a measure of carbamylation at lysine residues (12). Another portion of the dissolved protein was treated with an equal volume of 12 N HCl for 1 hr at 100°C. By this procedure, the carbamylated NH_2-terminal residues are converted to hydantoins. The subsequent identification of the hydantoins was performed as described by Stark and Smyth (12).

Preparation of cell lysates

Cell lysates were prepared after the cells were washed three times with PBS; the washed cells were then lysed by the addition of an equal volume of water. The stroma were removed by centrifugation at 10,000 × *g* for 10 min at 4°C. The lysates were concentrated by vacuum dialysis at 4°C to a hemoglobin concentration of 150–200 mg/ml. Deoxygenated HbS was stored at 4°C.

Gelation of deoxy HbS at 37°C was judged to be complete when the solution did not flow upon inversion of the tube.

RESULTS

Effects of KCNO and urea on the sickling of erythrocytes

Low concentrations of KCNO inhibit the sickling of sickle-cell erythrocytes as shown by the micrographs in Fig. 1. The morphology of deoxygenated erythrocytes without added cyanate is abnormal (Fig. 1*b*), whereas the proportion of normal deoxygenated cells is increased in the cyanate-treated sample (Fig. 1*c*).

High concentrations of urea also inhibit the sickling of erythrocytes, but urea differs from KCNO in that its effect is reversible (Table 1). The cells that were both incubated and deoxygenated in 1 M urea are inhibited from sickling (Expt. 2); however, the effect of the 1 M urea is abolished if the cell suspension is diluted to a final concentration of 0.1 M urea before deoxygenation (Expt. 4). KCNO (Expt. 6) inhibits sickling to the same degree as 1 M urea, but this inhibition is not affected by dilution. In fact, when the cyanate-treated cells are dialyzed or washed (Table 2) the sickling is still inhibited.

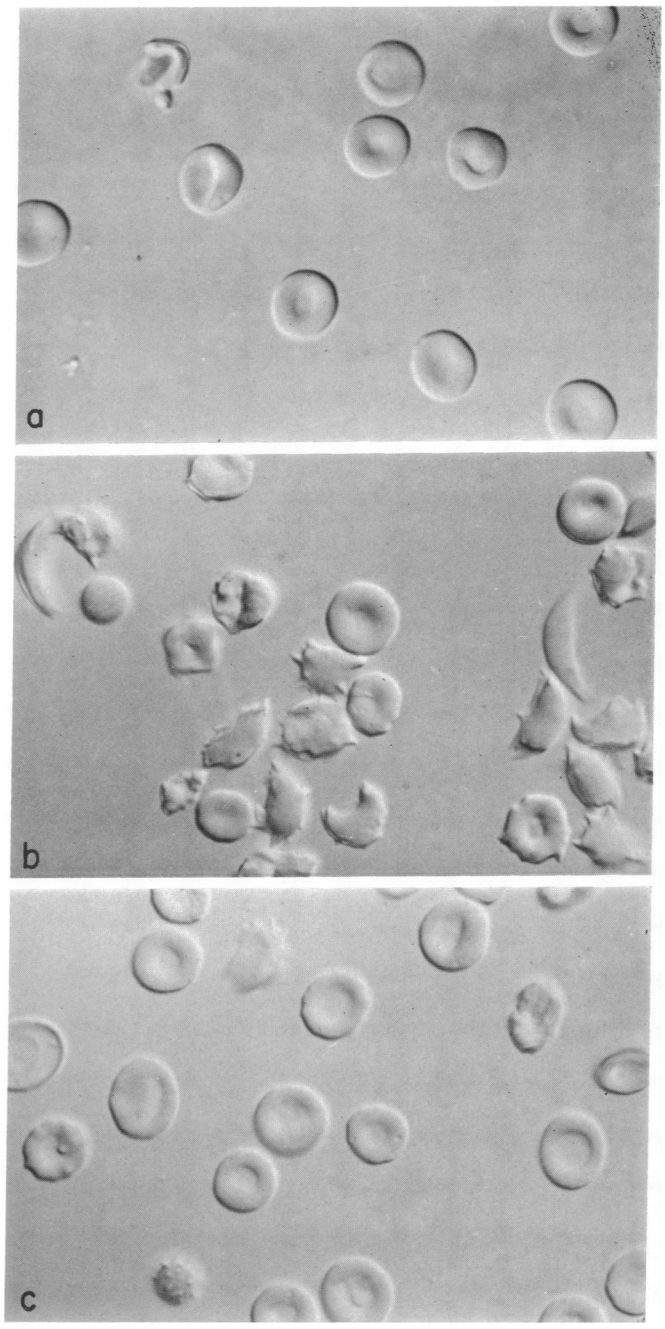

FIG. 1. (*a*) Oxygenated sickle-cell erythrocytes. (*b*) Deoxygenated sickle-cell erythrocytes. (*c*) Deoxygenated sickle-cell erythrocytes that had been treated in the oxygenated state with KCNO (0.03 M) for 1 hr at 37°C. The micrographs were kindly taken by Dr. James Jamieson, Rockefeller University, with a Zeiss microscope with Nomarski differential interference contrast optics (×800).

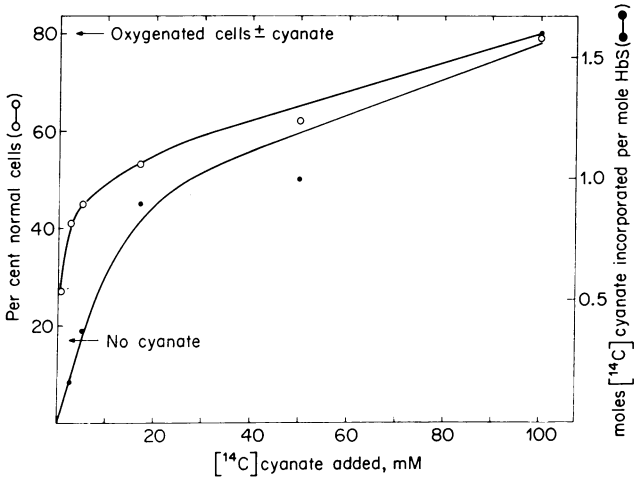

FIG. 2. The effect of KCNO on deoxygenated sickle-cell erythrocytes. Suspensions of oxygenated erythrocytes (0.5 ml) were incubated at 37°C with the designated amount of $K^{14}CNO$ (3.5×10^4 dpm/μmol). At the end of 1 hr, aliquots were removed for deoxygenation and determination of radioactivity.

The percentage of normal oxygenated cells was the same (80%) in the presence or absence of KCNO; the remaining 20% of these cells are irreversibly sickled (14), i.e., they have abnormal morphology after oxygenation. Upon deoxygenation, 17% of the cells have normal morphology.

The increase in the proportion of morphologically normal deoxygenated sickle-cell erythrocytes is directly related to the amount of cyanate incorporated. This was shown either by incubation of the cells for a fixed time with increasing concentrations of KCNO (Fig. 2) or by incubation of the cells at a fixed concentration of KCNO as a function of time (Fig. 3). We have determined dose–response curves for the erythrocytes from six other patients and have observed similar relationships; however, the amount of incorporated cyanate necessary to prevent 50% of the cells from sickling varies from 0.1 to 1 mol of cyanate per mol of hemoglobin. The reason for the 10-fold range in response with erythrocytes from various patients is unknown.

When deoxygenated sickled erythrocytes are incubated with 0.1 M $K^{14}CNO$ (Table 3), the same amount of radioactivity is incorporated as in the oxygenated cells, but the sickling is not reversed. When such cells are oxygenated, normal morphology is restored. Upon subsequent deoxygenation the cyanate-treated cells no longer sickle.

TABLE 2. *Effect of dialysis and washing on the sickling of cyanate-treated erythrocytes**

Expt.	Cyanate (M)	Dialysis	Normal deoxygenated cells (%)
1	—	—	17
2	—	+	14
3	0.05	—	62
4	0.05	+	63
		Washing	
5	—	—	16
6	0.04	—	57
7	0.04	+	59

Oxygenated erythrocytes were incubated for 1 hr at 37°C. In Expts. 2 and 4, the cell suspensions were dialyzed for 20 hr against 500 volumes of PBS at 4°C. In Expt. 7 the cells were washed three times with 22 volumes of PBS. The cells were diluted with PBS and deoxygenated as described in *Methods*.

* An oxygenated sample had 89% normal cells.

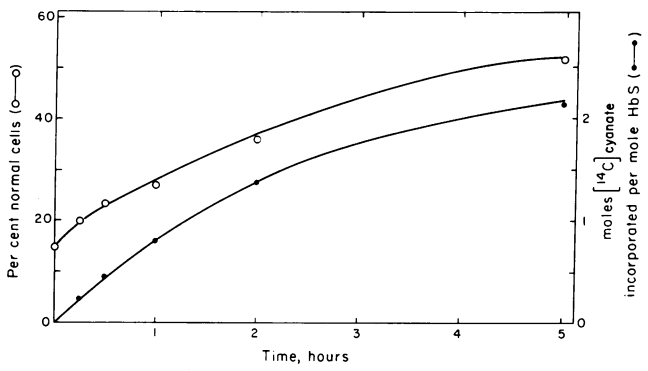

FIG. 3. The kinetics of carbamylation and the increase in normal deoxygenated sickle-cell erythrocytes. Suspensions of oxygenated erythrocytes (2.0 ml) were incubated at 37°C with 0.01 M $K^{14}CNO$ (3.1×10^4 dpm/μmol). At the indicated times, aliquots were removed for deoxygenation and determination of radioactivity.

The carbamylation of HbS

Disc gel electrophoresis (15) of erythrocyte protein from cells that had been incubated with $K^{14}CNO$ showed that all of the radioactivity was in the HbS band. The site of carbamylation on HbS was determined by amino acid analyses of hydrolysates of the sample that contained 1.6 mol of cyanate per mol of hemoglobin (Fig. 2). Alkaline hydrolysis of the isolated hydantoin fraction (12) yielded only valine (1.4 mol per mol of hemoglobin). Homocitrulline was absent from an acid hydrolysate of another portion of the sample; this indicates that there is no detectable carbamylation of the lysine residues. In another experiment, cells (from another patient) that were incubated with 0.01 M $K^{14}CNO$ incorporated 1.5 mol of cyanate per mol of hemoglobin. Isolation of the hydantoin fraction yielded 1.2 mol of valine per mol of hemoglobin. Since CO_2 also reacts with the NH_2-terminal residues of Hb to form carbamino compounds (16, 17), the reactive tautomer of cyanate, HN=C=O (isocyanic acid), may act as an analog of O=C=O.

TABLE 3. *Effect of KCNO on deoxygenated sickle cells*

	% Normal cells	
	Without cyanate	With cyanate
First deoxygenation	14	11
Reoxygenation	65	65
Second deoxygenation	19	61

The cells (0.5 ml) were placed in a tube with or without $K^{14}CNO$ (0.1 M) in the side arm; an oxygenated sample had 88% normal cells. After evacuation, as described in *Methods*, the contents of the side arm were mixed with the cells and the solution was then incubated for 1 hr at 37°C. A portion was fixed with formalin before and after oxygenation. The cells were deoxygenated again after dilution with 9 volumes of PBS and a portion was fixed with formalin.

The deoxygenated cells incorporated 1.80 carbamyl groups per hemoglobin molecule; oxygenated cells incorporated 1.68 carbamyl groups per hemoglobin molecule.

TABLE 4. *Effect of KCNO and urea on the gelling of hemoglobin S*

Addition	Concentration (mM)	Gelling
—	—	+
KCNO	1	−
Urea	10	+
Urea	50	+
Urea	100	−

Oxygenated hemoglobin S (200 mg/ml; 0.2 ml) was incubated with KCNO or with urea for 1 hr at 37°C. The contents of the tubes were gassed with 90% N_2–10% CO_2 for 5 min at 0°C. The tubes were incubated at 37°C; after 3 hr the presence or absence of a gel was determined.

We cannot exclude a small amount of reversible carbamylation at cysteine residues from these data (18). However, two findings argue against this possibility: (*a*) the specific activity of ^{14}C-labeled cells does not decrease after 24 hr at 37°C, and (*b*) the recovery of radioactivity that corresponds to carbamylated valine is nearly quantitative.

Spectrophotometric measurements indicate that there is no detectable liganding of cyanate with HbS, nor is there observable formation of methemoglobin.

Inhibition of hemoglobin S gelation

In contrast to deoxyhemoglobin A, deoxyhemoglobin S has the unusual property of reversible gel formation at 37°C (19, 20); the gel redissolves at 0°C (21). We have used this property to test the relative efficacy of KCNO and urea on the inhibition of gel formation. The results in Table 4 indicate that the gelation of HbS can be prevented by KCNO at a concentration that is 1% that of urea. The concentration of KCNO needed to prevent gel formation varies with the preparation of HbS; another preparation required about 2.5 mM KCNO to inhibit gel formation.

We have also tested the effect of KCNO on deoxygenated hemoglobin S. An evacuated tube contained a gel of deoxygenated hemoglobin S with KCNO in the side arm. The gel was liquified by lowering the temperature to 0°C and the cyanate and the protein solution were then mixed; the incubation at 37°C was resumed immediately. Gel formation was prevented by a concentration of 20 mM KCNO.

DISCUSSION

A molar solution of urea at 37°C and pH 7.4 contains, at equilibrium, 5 mM cyanate (22). The time necessary for the attainment of the equilibrium concentration of cyanate from urea depends upon the concentration of urea, pH, temperature of dissolution of the urea, and the temperature of storage of the solution (23). Thus, solutions of urea prepared for infusion may contain different amounts of cyanate. The concentration of cyanate necessary to have an effect on sickling *in vivo* could be less than that *in vitro* if we assume a cumulative reaction over a longer period of time. For these reasons, we cannot determine the extent to which cyanate might have been responsible for the reported effectiveness (1–4) of urea treatment in the crisis phase of sickle-cell anemia.

We have started studies to determine the effect of cyanate on various erythrocyte functions. Preliminary experiments indicate that cyanate-treated sickle-cell erythrocytes (one carbamyl group per hemoglobin molecule) have a normal capacity to bind and release O_2 (manuscript in preparation). Horse Hb that contains four carbamyl groups on the NH_2-terminal valine residue also has a nearly normal oxygen-dissociation curve (17). Another important function of Hb is the transport of CO_2 as carbamino groups on the NH_2-terminal valine residues of the protein (16). Since the presence of carbamyl groups on these NH_2-terminal valine residues of Hb will prevent the formation of carbamino compounds (17), we have tested whether blocking of some of the NH_2-terminal valine residues is detrimental *in vivo*. We have injected mice once daily with a sublethal dose of KCNO (28 μmol per mouse) without any apparent ill effects. After 20 injections, the mouse erythrocytes contained an average of 1.6 carbamyl groups per hemoglobin molecule. Thus, the presence of several carbamyl groups on hemoglobin does not seem to interfere seriously with the physiology of the red blood cell; this result suggests the possibility that cyanate may be used to prevent erythrocyte sickling *in vivo*.

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