

Inhibition of Erythrocyte Sickling In Vitro by Pyridoxal

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ABSTRACT To test the antisickling activity of pyridoxal, we compared the oxygen affinity and the percent sickling at low PO_2 of untreated erythrocytes with values for cells from the same blood sample incubated with pyridoxal, glyceraldehyde, or pyridoxine. Pyridoxal increased oxygen affinity much more than glyceraldehyde. 20 mM pyridoxal and glyceraldehyde had equivalent antisickling activity. At PO_2 levels above 20 mm Hg, both agents reduced sickling to less than 2%. In samples examined by electron microscopy, pyridoxal reduced the percent sickled cells and the percent cells that contain hemoglobin S fibers by the same amount (from 74 to 3%). Pyridoxine had no effect on oxygen affinity or sickling. Pyridoxal reacts with intracellular hemoglobin to increase oxygen affinity, which inhibits hemoglobin S polymerization and sickling.

INTRODUCTION

Erythrocyte sickling is initiated by the polymerization of deoxyhemoglobin S. Although several derivatives of pyridoxal react with hemoglobin (Hb)¹ and inhibit polymerization of Hb S (1–3), none appear promising for clinical use because they are either B₆ antagonists (e.g., deoxypyridoxal) or they contain polar side chains which limit entry into the erythrocyte (e.g., pyridoxal phosphate). In contrast, pyridoxal is a form of vitamin B₆ which is actively transported into erythrocytes (4). However, pyridoxal appeared unreactive (5) or reacted so slowly with a dilute solution of Hb S (3) that it was not tested for antisickling activity. Only 10% of Hb S

was modified after erythrocytes were incubated with 3 mM pyridoxal (6). In this experiment a large increase in oxygen affinity was noted. This suggested to us that pyridoxal did react extensively with Hb, but the adduct was dissociated in preparing hemolysates for electrophoresis. We therefore examined the effect of pyridoxal on the oxygen affinity of intact erythrocytes and on the percent sickled cells that result from exposure to low PO_2 . Pyridoxal was compared to glyceraldehyde, another carbonyl compound which had defined antisickling activity (7, 8), and with pyridoxine, the alcohol analogue of pyridoxal.

METHODS

Materials. Pyridoxal hydrochloride, pyridoxine hydrochloride, DL-glyceraldehyde, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Heparin without preservative was obtained from Flow Laboratories, Rockville, Md. All other chemicals were reagent grade.

Preparation of treated erythrocytes. Venous blood was drawn with informed consent from donors who had a normal Hb phenotype (AA) or a phenotype consistent with homozygous Hb S (SS), (9). For investigation of antisickling effects, blood was drawn into anticoagulant citrate phosphate dextrose solution (CPD) and stored at 4°C for up to 6 days. Erythrocytes were washed three times and suspended at 10% hematocrit in phosphate buffer that consists of 145.4 mM NaCl, 4 mM KCl, 1.65 mM Na_2HPO_4 , 0.16 mM KH_2PO_4 , 11.1 mM D-glucose, and 50 mg/liter bovine serum albumin. Four reaction mixtures were prepared from each suspension; a control and mixtures with 20 mM pyridoxal, glyceraldehyde, or pyridoxine. The pH was adjusted to 7.4 and each mixture was adjusted to 290 ± 2 mosmol, excluding the compound tested. Reaction mixtures were incubated in a shaking water bath at 37°C in dim light for 90 min, transferred to shaking tonometers, and equilibrated for 30 min at 37°C with a humidified mixture of N_2 , O_2 , and CO_2 . The partial pressure of each gas was varied independently, with an Ohio Medical Products anesthesia machine with modified fittings (Ohio Medical Products Div., Madison, Wis.). Before and after equilibration, the pH and PO_2 were measured in a Corning 161 Blood Gas Analyzer (Corning Medical, Corning Glassworks, Medfield, Mass.) and cells were drawn anaerobically into cold 1% buffered glutaraldehyde at pH 7.4. To eliminate the effect of CPD or buffer on erythrocyte oxygen affinity, this ex-

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¹Abbreviations used in this paper: AA, normal hemoglobin phenotype; CPD, anticoagulant citrate phosphate dextrose solution; Hb, hemoglobin; P_{50} , the PO_2 at which hemoglobin is half saturated; SS, homozygous hemoglobin S phenotype.

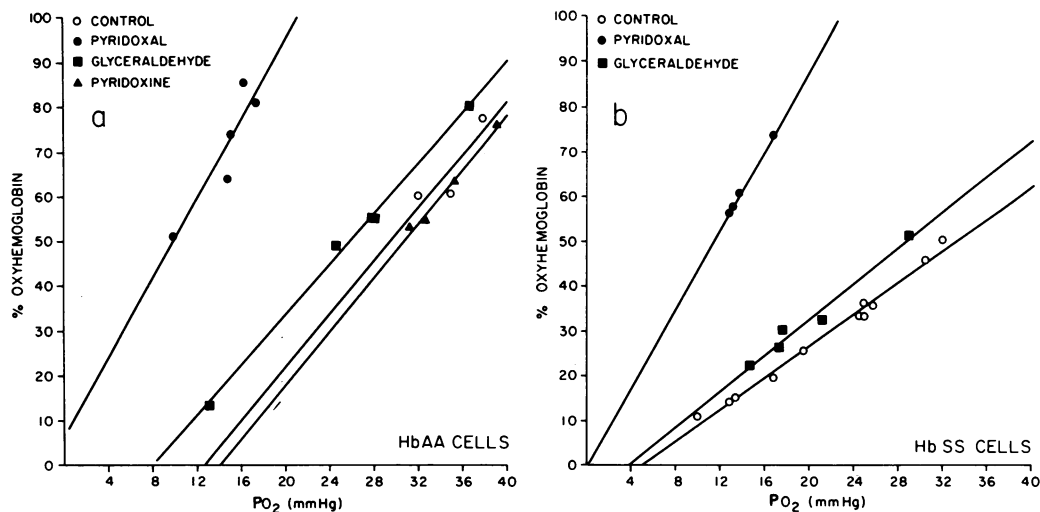


FIGURE 1 Effect of three compounds on the oxygen affinity of whole blood. Points on the linear part of the oxygen dissociation curve were determined after incubation of whole blood untreated or with 20 mM compound added: (a) a single AA blood sample; (b) a single SS blood sample.

periment was repeated with fresh, heparinized whole blood from four SS patients.

Effects on oxygen affinity. Fresh, heparinized whole blood from two AA and two SS individuals was incubated and exposed to low PO_2 as described above. The percent oxy-Hb was measured with an IL 182 Co-Oximeter (Instrumentation Laboratory, Inc., Lexington, Mass.) to define the linear portion of the oxygen dissociation curve for each reaction mixture. The PO_2 at which Hb was half saturated (P_{50}) was calculated from a linear regression.

Effects on sickling. Two observers counted from 250 to 500 fixed erythrocytes from each reaction mixture with an AO H21 interference microscope (C. Reichert, sold by American

Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) with a $\times 100$ oil immersion objective. The observed variance of the proportion of sickle cells in each sample (as defined by counting subgroups of 50 cells) was close to that predicted by the binomial distribution. Slides with greater variation were prepared again. The number of cells counted was chosen to identify a 20% decrease in sickling with a false negative rate <0.05 and a false positive rate <0.01 (10). Erythrocytes were considered reversibly sickled if the cells were spiculated and elongated or had a transparent veil of cytoplasm along one side (11, 12).

Fixed erythrocytes from 20 experiments were also examined and photographed in an AMR-1000 scanning elec-

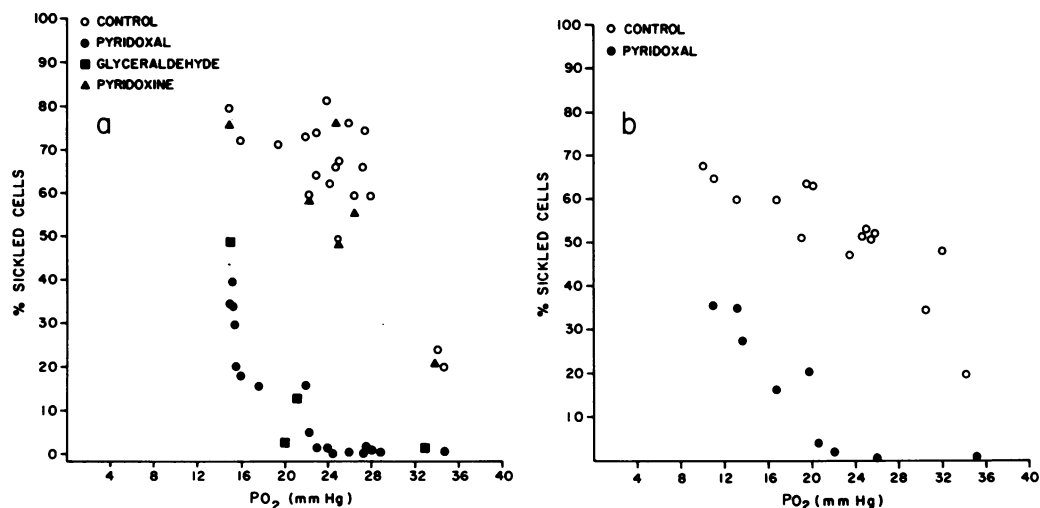


FIGURE 2 Effects of three compounds on erythrocyte sickling at varied oxygen tensions. Erythrocytes were incubated 90 min at 37°C , untreated or with 20 mM compound, equilibrated at low PO_2 for 30 min, fixed, and examined. (a) Studies of cells from eight SS patients with stored, washed cells resuspended in buffer as described in Methods. (b) Studies of fresh, whole SS blood from four patients.

tron microscope (AMR Corp., Bedford, Mass.) (13). Cells from three experiments were prepared for transmission electron microscopy (14). Thin sections were stained with lead citrate and uranyl acetate, examined, and photographed in an AEI EM 8 electron microscope (AEI Scientific Instruments, San Diego, Calif.).

RESULTS

Effects on whole blood oxygen affinity. The effects of pyridoxal, glyceraldehyde, and pyridoxine on the oxygen affinity of whole blood are shown in Fig. 1 and analyzed in Table I. Pyridoxal produced a much greater left shift in the oxygen dissociation curves than glyceraldehyde. Pyridoxine had no effect on oxygen affinity.

Effects on erythrocyte sickling. The relation between percent sickling and PO_2 was determined for erythrocytes drawn in CPD and suspended in buffer (Fig. 2a). Pyridoxine had no effect on sickling. Plots for pyridoxal and glyceraldehyde were identical, with significant reduction in sickling (pyridoxal, $P < 0.001$; glyceraldehyde, $P < 0.03$, the sign test). In paired experiments on blood from five patients, the antisickling activity of pyridoxal (percent sickling, mean \pm SD, $7.8 \pm 6.4\%$) and glyceraldehyde ($19.2 \pm 20.4\%$) were not significantly different ($P < 0.3$, paired t test). The relation between percent sickling and PO_2 was not altered by use of heparinized whole blood (Fig. 2b) instead of erythrocytes in buffer. The following values were observed for blood from four SS patients treated with 5 mM instead of 20 mM pyridoxal: 0.6% sickling at a PO_2 of 35.2 mm Hg, 1.3% at 26.3 mm Hg, 42% at 23 mm Hg, and 64% at 15 mm Hg. Sickling of untreated samples was 20, 81, 65.5, and 79.3%, respectively.

Scanning electron micrographs of representative samples (Fig. 3) confirmed the differences in degree of sickling noted by interference microscopy.

Effects on Hb S fiber formation. In transmission electron micrographs, untreated samples had a much higher proportion of cells that contain intracellular fibers characteristic of Hb S polymers (12) than did

pyridoxal treated samples. A close correlation was found between the percent cells that contain Hb S fibers and the percent sickle forms by light microscopy (untreated cells: 75% Hb S fibers, 72.6% sickled; pyridoxal treated cells: 2.9% Hb S fibers, 2.4% sickle forms) (Figs. 3 and 4).

DISCUSSION

This report provides evidence that pyridoxal inhibits sickling of SS erythrocytes by modification of Hb S. Pyridoxal reduced the fraction of cells that contain Hb S fibers at low PO_2 from 75% to 3%. A close correspondence was found between the percent sickled cells and the percent cells that contain Hb S fibers. Pyridoxal

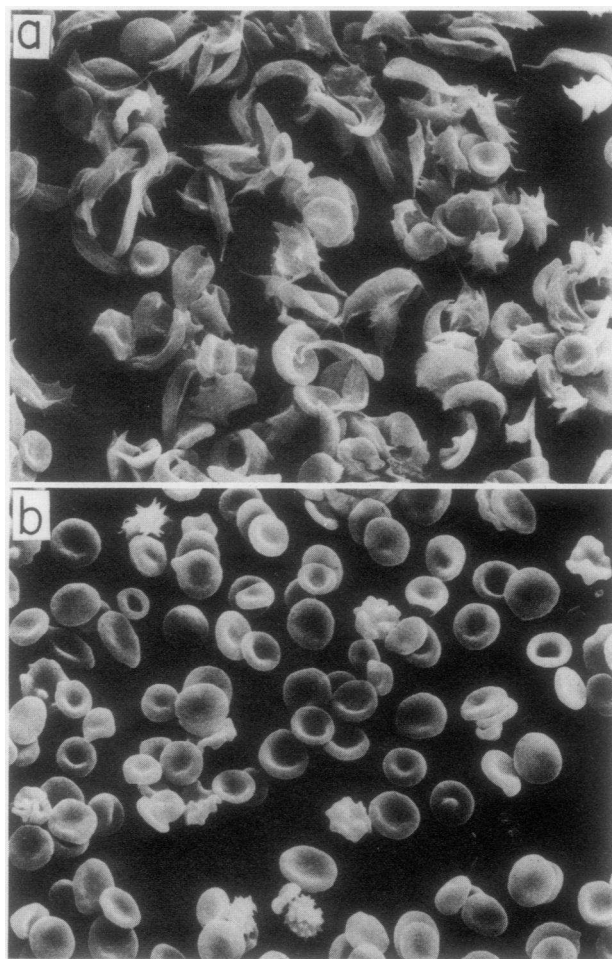


FIGURE 3 Effect of pyridoxal on erythrocyte sickling. Washed erythrocytes from a single SS blood sample were suspended in buffer, incubated untreated or with 10 mM pyridoxal, equilibrated at a PO_2 of 23 mm Hg, and fixed. Scanning electron micrographs at $\times 2,000$ magnification show: (a) untreated cells with about 70% spiculated sickle forms; (b) pyridoxal treated cells with more than 90% discocytes, occasional echinocytes, and about 1% sickle forms.

TABLE I
 P_{50} Values for Normal and Sickle Cell Blood

Agent	P_{50}	
	AA blood	SS blood
	mm Hg	
Untreated	29.5	33.1
Pyridoxal, 20 mM	9.9	11.4
Glyceraldehyde, 20 mM	25.7	28.6
Pyridoxine, 20 mM	30.6	—

The P_{50} was calculated from the linear regressions shown in Fig. 1.

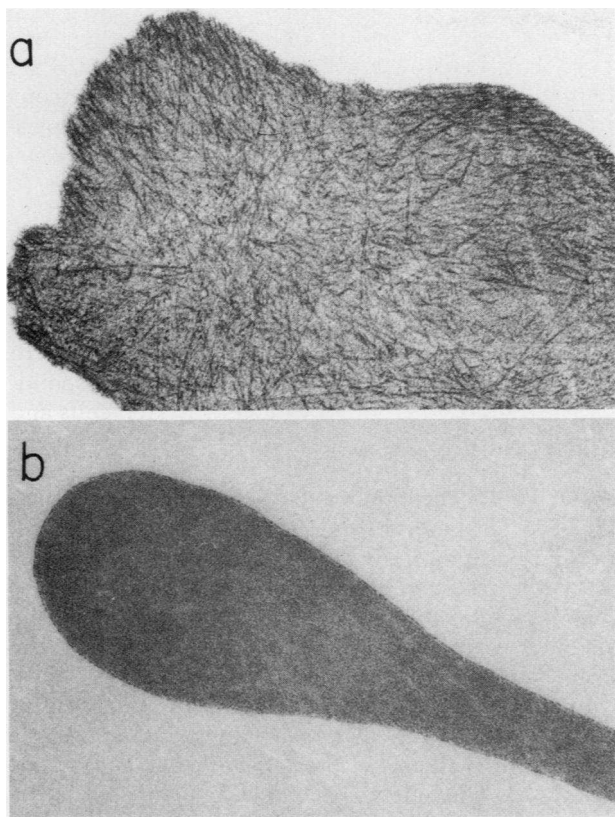


FIGURE 4 Effect of pyridoxal on intracellular fibers. Fixed erythrocytes from the experiment shown in Fig. 3 were sectioned and examined by transmission electron microscopy at $\times 40,000$ magnification. (a) Portion of an untreated cell showing intracellular Hb S fibers; (b) Portion of pyridoxal-treated cell, which shows absence of Hb S fibers.

reduced the P_{50} of SS or AA blood by 67%, as reported for AA blood (6). The increase in proportion of oxy-Hb was adequate to explain the antisickling activity of pyridoxal. However, a low P_{50} might complicate the clinical use of pyridoxal by an adverse effect on oxygen delivery and by compensatory erythropoiesis. In contrast, glyceraldehyde did not substantially decrease the P_{50} . Different amino acid residues of Hb are modified by glyceraldehyde (8) and pyridoxal derivatives (2, 3, 15). Therefore, it might be possible to modify Hb with both glyceraldehyde and pyridoxal, perhaps inhibiting sickling with lower doses of each compound.

20 mM pyridoxal and glyceraldehyde had similar antisickling activity. Studies of control and pyridoxal-treated heparinized whole blood demonstrated that artifacts caused by the storage or processing of erythrocytes were not important. Single observations on blood from four patients showed significant antisickling activity for 5 mM pyridoxal, comparable to published data for deoxypyridoxal (3), *o*-vanillin (6), and salicylaldehyde (6). These results suggest that the antisickling

activity of pyridoxal may be equivalent to the activity of these three agents and greater than that of cyanate (6).

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