

Pyridoxal Phosphate as an Antisickling Agent In Vitro

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ABSTRACT Although pyridoxal phosphate is known to inhibit gelation of purified hemoglobin S, antisickling activity has never been demonstrated for intact erythrocytes. We incubated washed erythrocytes at 37°C either in buffer alone, or with added pyridoxal phosphate or pyridoxal, washed these cells, suspended them in untreated buffer, and compared the percent modified hemoglobin, the oxygen affinity, and the extent of sickling under hypoxia. Pyridoxal phosphate modified intracellular hemoglobin more slowly than pyridoxal. Pyridoxal phosphate lowered the oxygen affinity of normal cells, but had no effect on oxygen binding by sickle cells. Pyridoxal increased the oxygen affinity of normal and sickle erythrocytes equally. Pyridoxal phosphate significantly inhibited sickling of sickle or sickle trait erythrocytes ($P < 0.001$). Inhibition of sickling by pyridoxal phosphate was largely independent of oxygen binding; whereas inhibition of sickling by pyridoxal was almost entirely dependent on increased oxygen binding. Although pyridoxal phosphate and pyridoxal both inhibit sickling by modification of hemoglobin S, they differ in the kinetics of whole cell modification, the effect on oxygen affinity of intact cells, and the mechanism of action of the antisickling activity.

INTRODUCTION

Studies of hemoglobin (Hb) solutions have demonstrated that pyridoxal 5'-phosphate (PLP),¹ the major

transport and coenzyme form of vitamin B₆, binds to Hb by reversible formation of Schiff bases between its 4'-aldehyde and the free amino groups of Hb (1). Reaction of PLP with purified deoxy-Hb under nitrogen produced selective modification of the β NH₂-termini of Hb (2). This modification reduced the oxygen affinity of Hb (1, 2) and raised the minimum gelling concentration of solutions of deoxy-Hb S (3). Thus PLP may function as an antisickling agent that would not compromise the transport of oxygen by modified Hb. However, fresh erythrocytes in plasma failed to take up enough PLP for detection of PLP-Hb adducts (4). When normal erythrocytes were preincubated for 12 h at 37°C, washed, and suspended in isotonic phosphate buffer, incubation with PLP for several hours produced substantial modification of Hb (5). Unfortunately, this process seems too complex for clinical use.

We became interested in the potential use of PLP and its precursor, pyridoxal, as antisickling agents because these forms of vitamin B₆ are widely distributed in normal tissues and have low toxicity (6, 7). Since pyridoxal rapidly enters intact erythrocytes when added to whole blood, it was easy to demonstrate inhibition of erythrocyte sickling in vitro (8). In preparation for similar studies with PLP, we determined the optimum conditions for the formation of PLP-Hb and pyridoxal-Hb in normal and sickle cells (9). Sickle erythrocytes could be adequately loaded with PLP by incubating fresh, washed cells at 37°C for 30–40 min in reaction mixtures containing 30 mM PLP. Preincubation of the blood and subsequent use of isotonic phosphate buffer for the reaction mixtures were not required. This simple process appears practical for extracorporeal treatment of patients.

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

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¹ Abbreviations used in this paper: AA, normal hemoglobin phenotype; ACD, anticoagulant acid citrate dextrose solution; AS, sickle cell trait hemoglobin phenotype; MCHC, mean cell hemoglobin concentration; P₅₀, the PO₂ at which

hemoglobin is half saturated; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; S/ β^0 thalassemia, double heterozygous hemoglobin S and β^0 thalassemia phenotype, SC, double heterozygous hemoglobin SC phenotype; SS, homozygous hemoglobin S phenotype.

This report compares the properties of fresh erythrocytes, washed and incubated with PLP, with cells from the same sample that were incubated with pyridoxal or with untreated buffer. Echinocyte transformation, hemoglobin modification, oxygen affinity, and the extent of sickling as a function of PO_2 or percent of oxy-Hb were examined to assess the behavior of pyridoxal phosphate and pyridoxal as antisickling agents.

METHODS

Materials and instrumentation. PLP, pyridoxal hydrochloride, pyridoxamine 5'-phosphate (PMP), glutaraldehyde, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. The percent Hb as PLP-Hb or pyridoxal-Hb was determined by high-performance liquid chromatography on an ion exchange resin of paired treated and control hemolysates (9). Blood gas measurements were performed with a Corning 165 Blood Gas Analyzer (Corning Scientific Instruments, Medfield, MA). The percent oxy-Hb was determined with an IL 282 Co-oximeter (Instrumentation Laboratory, Inc., Lexington, MA) and IL 237 tonometers were used for equilibration of cells with gas mixtures.

Preparation of treated erythrocytes. After obtaining informed consent, venous blood was drawn into heparin or acid citrate dextrose anticoagulant (ACD) from five donors with a normal Hb phenotype (AA), five with homozygous Hb S phenotype (SS), three with Hb SC phenotype (SC), and two donors with sickle trait phenotype (AS) (9). Paired samples of control and treated erythrocytes were prepared using a control buffer (8) modified to contain 24 mM $NaHCO_3$, and treated buffers containing 45 mM PLP or 22.5 mM pyridoxal (8, 9). These buffers were kept at a PO_2 of 100 torr and a pH of 7.4 ± 0.05 by equilibration in tonometers with a humidified mixture of N_2 , O_2 , and CO_2 (8). Treated cells were examined by interference microscopy (8) and washed six times with normal saline. Aliquots were taken to measure the percent modified Hb (9) and to obtain the oxygen association curve for cells in autologous plasma, using the Hemo-O-Scan (American Instrument Co., Columbia, MD). To follow the rapid changes in oxygen affinity caused by pyridoxal, whole heparinized blood was equilibrated at $37^\circ C$ with the gas phase at a PO_2 of 15 or 26 torr. The percent oxy-Hb was determined with a cooximeter before and after addition of buffered pyridoxal (8).

Paired aliquots of washed treated and control erythrocytes from each donor were suspended in control buffer and stored at $4^\circ C$. Paired aliquots of cells were equilibrated in tonometers at $37^\circ C$ for 1 h with humidified N_2 , O_2 , and CO_2 to obtain the desired PO_2 and to stabilize the pH at 7.4 ± 0.05 . (8). Samples were then fixed in glutaraldehyde, or taken for blood gas analysis, measurement of percent oxy-Hb, and measurement of percent modified Hb (8, 9). Two observers determined the percentage of reversibly sickled cells for each sample, using interference microscopy to evaluate cell shape (8). Each observer classified 250 or 500 cells in subgroups of 50 cells. The observed variance in percent sickled cells of the subgroups was close to that predicted by the binomial distribution. If a set of counts exhibited greater than predicted variance, the sample was reevaluated from a fresh slide. The number of cells counted was chosen to identify a 10% decrease in sickling with a false negative rate <0.05 and a false positive rate <0.01 . Variability between

observers was not significant by Student's paired t test, as confirmed by a mean difference for sickling rates between observers of 0.2% ($n = 29$) and a mean SE for combined subgroup counts on the same 29 samples of 1.4%.

To determine whether PLP might reduce sickling by a mechanism unrelated to the modification of Hb S, such as an increase in erythrocyte hydration, similar experiments were performed using blood from 10 patients with sickle cell anemia (seven with SS, two with SC, and one with S/ β^0 thalassemia phenotype). The extent of sickling at low PO_2 , the mean cell hemoglobin concentration (MCHC), and the cell size distribution were compared for cells preincubated in control buffer, in a reaction mixture with 30 mM PMP (an analog of PLP that does not bind to Hb), or in 30 mM PLP. After washing the cells, equilibrating them in control buffer at a PO_2 from 30 to 70 torr, and assessing extent of sickling, the MCHC of the cells was determined by triplicate measurement of the reaction mixture hematocrit, obtained by centrifugation of capillary tubes, and the hemoglobin concentration, obtained by spectrophotometry of aliquots added to Drabkin's solution. Cell size distribution was examined on aliquots of glutaraldehyde-fixed cells, using a ZH Coulter Counter linked to a C-1000 Coulter Channelyzer (Coulter Electronics Inc., Hialeah, FL). Since no changes in the shape of the volume distribution curves were noted, cell volume was evaluated from the median cell size. Median cell size was calculated by linear extrapolation between the exclusion volumes for the two channels that bracketed one-half of the total cell count, using a total count of $\sim 50,000$ cells.

RESULTS

Erythrocyte oxygen affinity. When AA erythrocytes were incubated with PLP for 6 h, the mean P_{50} increased slowly relative to the appearance of PLP-Hb (Table I). The mean P_{50} of SS or SC erythrocytes did not change after 6 h of treatment with PLP, either using the Hem-O-Scan or selected points on the oxygen dissociation curve, obtained by tonometry and use of a cooximeter. In contrast, pyridoxal produced its maximum effect on the P_{50} of whole blood in <10 min. Furthermore, the absolute change in P_{50} , a fall of 19 torr, was similar for AA and SS cells, as previously reported (8).

TABLE I
Alteration in Hemoglobin of AA vs. SS Cells.
Changes in Intracellular Hemoglobin during Incubation at
 $37^\circ C$ of Intact AA and SS Erythrocytes in 30 mM PLP

Hours incubated	Percent PLP-Hb		P_{50}	
	AA	SS	AA	SS
0	0	0	26	34
1	20	46	27	34
2	37	48	30	34
3	43	53	32	34
4	58	59	36	34

Erythrocyte morphology in room air. Incubation of normal or sickle erythrocytes with PLP at 37°C caused extensive echinocyte formation within 15 min, even at concentrations of PLP as low as 0.5 mM. However, after washing cells five times to remove PLP and incubating them at 37°C in control buffer, reversion to discocytes was complete within 10 min. Echinocytes reverted just as completely for erythrocytes containing 50 to 70% PLP-Hb after washing, as for those with no detectable PLP-Hb. Pyridoxal did not alter the morphology of normal or sickle erythrocytes in room air.

Inhibition of sickling by PLP. The extent of sickling as a function of PO_2 was evaluated for paired control and PLP-treated SS, SC, or AS erythrocytes, equilibrated with the same gas mixture in the absence of external PLP. Erythrocytes were incubated for 1–5 h with PLP and contained 42–62% PLP-Hb. PLP consistently inhibited sickling of SS and SC cells (Fig. 1). PLP also reduced sickling of AS cells from two individuals after exposure of these cells to a $PO_2 < 5$ torr (percent sickled cells: for controls, 60 and 45%; for PLP-treated cells, 30 and 24%, respectively). The difference between the extent of sickling for the control and treated sample pairs was highly significant ($P < 0.01$ for each SC sample pair and $P < 0.001$ for each SS or AS sample pair, using Student's paired t test). Echinocyte formation did not contribute to this difference: $<0.4\%$ echinocytes were present in any sam-

ple. In addition, transient formation of echinocytes had no effect on the final extent of sickling for control cells that were exposed to PLP long enough for complete echinocyte transformation but too briefly for significant modification of intracellular Hb (V.S. and C.N., Fig. 1, SS cells).

Inhibition of sickling was dose related. In five experiments, each using erythrocytes from different patients with sickle cell disease, the reduction in sickling correlated with the concentration of PLP during treatment of the cells (percent sickled cells, mean \pm SE: $34 \pm 5.4\%$ with no PLP, $18 \pm 3.6\%$ with 15 mM PLP, and $8 \pm 1.7\%$ with 30 mM PLP, $P < 0.025$ for all sequential pairs). PLP was more effective when the whole blood was drawn into heparin than when it was drawn into ACD. In paired experiments with cells drawn into each anticoagulant from the same five patients, the anti-sickling activity of PLP was compared. The mean percent sickling was virtually identical for untreated cells drawn in either anticoagulant (mean \pm SE: $36 \pm 5\%$ for cells drawn in heparin vs. $35 \pm 6\%$ for cells drawn in ACD). After treatment with PLP, the mean (\pm SE) percent sickled cells was lower for cells drawn in heparin ($2 \pm 1\%$) than for cells drawn in ACD ($13 \pm 4\%$, $P < 0.01$, by Student's paired t test). However, the percent PLP-Hb was identical for the cells drawn in each anticoagulant. Inhibition of sickling also depended on the duration of exposure of cells to PLP at

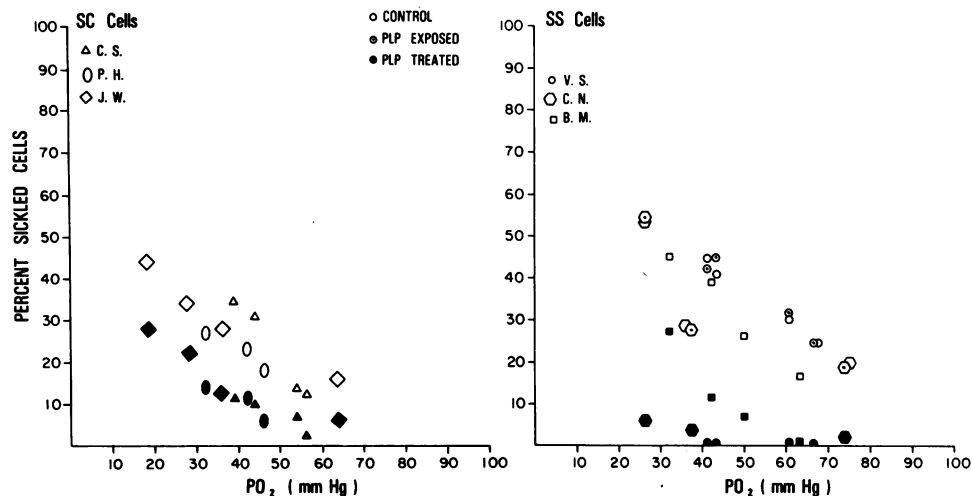


FIGURE 1 Effect of PLP on erythrocyte sickling as a function of PO_2 . SS or SC cells were incubated in control and 30-mM PLP reaction mixtures. Control and treated sample pairs were washed, suspended in untreated buffer, equilibrated with the same gas mixture, sampled to measure the PO_2 , fixed and evaluated for extent of sickling. Experiments on cells from V.S. and C.N. included an additional control (open figure with central dot); these cells were transiently exposed to PLP without significant loading, by incubation with PLP for 15 min before washing and partial deoxygenation. Thus, these cells were echinocytic at the same time as the PLP-treated cells, but were deoxygenated without PLP-Hb being present, like the other control cells.

37°C. The minimum incubation time required to produce more than a 10% reduction in sickling was only 30 min for cells drawn in heparin, but was 3 h for cells drawn in ACD. For cells drawn in heparin, the reduction in sickling with PLP was maximal after a 1-h incubation.

In 10 experiments, erythrocytes from single patients with sickle cell anemia were examined at a low PO_2 (30–70 torr) to compare the effects of preincubation in control buffer, in 30 mM PMP, or in 30 mM PLP. The mean (\pm SD) MCHC was 34.8 ± 2.2 g/dl for the 10 sets of control cells, 35.9 ± 2.9 for the PMP-treated cells, and 35.5 ± 4.4 for the PLP-treated cells. The mean (\pm SD) median cell size was 87.2 ± 12.3 fl for the 10 sets of control cells, 86.0 ± 15.4 for cells treated with PMP, and 88.7 ± 13.1 for cells treated with PLP. There were no significant differences between the control cells and the treated cells for mean MCHC or median cell size ($P > 0.30$, using Student's paired *t* test). The mean (\pm SD) percent sickled cells was 16.9 ± 8.9 for the 10 control samples, 15.0 ± 6.7 for the samples treated with PMP, and 5.3 ± 5.9 for the samples treated with PLP. PLP produced a significant reduction in sickling ($P < 0.001$, using Student's paired *t* test), but PMP had no significant effect on sickling ($P > 0.30$).

Mechanisms of antisickling activity. Data from studies of three SS and two SC patients were combined to compare the effect of PLP and pyridoxal on erythrocyte sickling as a function of the percent saturation of Hb with oxygen (Fig. 2). Control and PLP-treated cells fixed at a single PO_2 were readily identified as paired points at the same position on the x-axis (Fig. 2A), indicating that treatment with PLP produced no

significant change in the percent oxy-Hb of SS or SC cells. PLP reduced the sickled cells by 25% at all levels of oxy-Hb. Thus, the antisickling activity of PLP was independent of oxygen binding by Hb S. In contrast, pyridoxal raised the percent oxy-Hb of SS and SC cells, so that control and treated pairs fixed at the same PO_2 were not easily identified (Fig. 2B). However, 13/15 pyridoxal-treated samples fell on the same curve relating percent sickling to percent oxy-Hb as the untreated controls. Thus, the antisickling activity of pyridoxal was almost entirely dependent on increased oxygen binding by Hb S.

DISCUSSION

Evaluation of the effects of pyridoxal phosphate on erythrocyte sickling became possible after conditions were found that permitted rapid entry of sufficient PLP into intact fresh erythrocytes (9). This report describes substantial reduction of sickling of SS, SC, and AS erythrocytes after treatment with PLP, washing, and exposure to low PO_2 . The antisickling activity of PLP was less than previously reported for pyridoxal (8), but greater than for cyanate (4, 10). This effect of PLP was directly related to the duration of treatment and the concentration of PLP used. Since antisickling activity was observed in the absence of echinocyte forms, after washing the cells, and with substantial modification of Hb S, it appears that PLP inhibited sickling by its reaction with Hb S, not its reaction with erythrocyte membrane proteins (11). Furthermore, PMP, an analog of PLP that does not bind to Hb (1), had no significant effect on erythrocyte

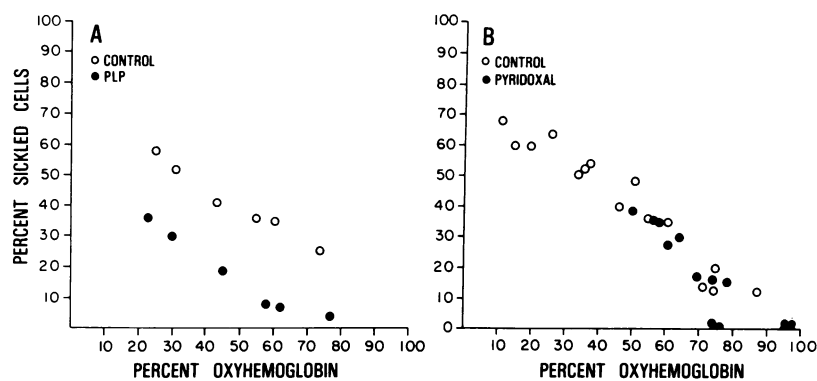


FIGURE 2 Effects of PLP and pyridoxal on sickling as a function of percent oxy-Hb. SS or SC cells were incubated in control and 30 mM PLP (A) or 15 mM pyridoxal (B) reaction mixtures. Control and treated sample pairs were washed, suspended in untreated buffer, equilibrated with the same gas mixtures, sampled to measure percent oxy-Hb, fixed, and evaluated for extent of sickling. Each point represents the mean values for samples from five subjects fixed at the same PO_2 after the same treatment. Sets of points for paired control and treated samples obtained at the same PO_2 are compared.

sickling. Prior treatment with PLP or PMP did not increase the hydration of sickle erythrocytes, since the MCHC and median cell size were similar to those of untreated control cells. However, the extent of modification of Hb was not the only factor determining the antisickling activity of PLP. Erythrocytes drawn in heparin were more sensitive to the antisickling effect of PLP than cells drawn in ACD, although both contained the same fraction of PLP-Hb.

Although the reversible nature of Schiff base formation may protect against potential toxicity from nonselective protein modification, this property also implies that hemoglobin modification may be transient *in vivo*. Previous studies of modified erythrocytes suggested that PLP adducts with Hb would be retained adequately *in vivo*, whereas pyridoxal adducts might have a rapid rate of dissipation (9). Pyridoxal and PLP also differed in the rate at which they modified intracellular Hb and in their effects on the oxygen affinity of AA and SS cells. Pyridoxal reduced the P_{50} of AA and SS cells equally (8) and this effect was maximal within 10 min of exposure of the cells at 37°C. In contrast, PLP steadily raised the P_{50} of AA cells over a period of 4 h. PLP had no effect on the P_{50} of SS or SC cells. The increase in P_{50} for molecules of Hb S that bound PLP (1, 2) was probably balanced by the fall in P_{50} for molecules of PLP released from polymers, an effect demonstrated previously with other antisickling agents (12–14). Thus, failure to observe an increased P_{50} for SS or SC cells was additional evidence that PLP inhibited polymerization of intracellular Hb S.

The molecular mechanisms by which pyridoxal derivatives alter the oxygen affinity of Hb A and the polymerization of Hb S have been elucidated by the formation of Schiff base adducts with purified Hb under conditions favoring the selective modification of the α - or β -NH₂-termini of Hb and subsequent reduction of these unstable adducts to the stable secondary amines (1, 2, 15). The use of pyridoxal derivatives with varied functional groups at the 5'-position of the pyridine ring and a modified form of PLP with a second aldehyde at the 2'-position has allowed detailed analysis of the effects of functional groups and attachment sites on alterations of Hb function (1, 2, 15, 16). PLP and its dialdehyde derivatives selectively bound to the β -NH₂-termini of deoxy-Hb. The 5'-phosphate group of these compounds stabilized deoxy-Hb by binding in the same positions as the phosphate groups of 2,3-diphosphoglycerate, resulting in a similar substantial increase in the P_{50} (1, 2, 15, 16). In fact, PLP formed doubly modified Hb with the two 5'-phosphate groups bound to Hb at the same sites as the two phosphate groups of 2,3-diphosphoglycerate (17). PLP inhibited

polymerization of purified Hb S in the absence of oxygen (3). When oxygenated SS or SC erythrocytes were treated with PLP, similar oxygen-independent inhibition of sickling was observed (Fig. 2). Although our experiments did not define the sites of modification of intracellular Hb by PLP, the effects of PLP on AA, SS, and SC erythrocytes were consistent with predominant formation of Schiff bases on the β -NH₂-termini of Hb. Nevertheless, it remains possible that attachment of PLP to other sites on Hb also increases the P_{50} and inhibits polymerization of deoxy-Hb S.

Of those compounds studied, pyridoxal is most like deoxypyridoxal in chemical structure, since both compounds have a small, relatively nonpolar 5' group. Both compounds bound to the α -NH₂-termini of Hb (15, 18, 19) and both inhibited sickling entirely by an oxygen-dependent mechanism (8, 14, 18, Fig. 2). It is interesting that cyanate exhibited oxygen-dependent and oxygen-independent inhibition of polymerization of Hb S, and these effects also correlated with binding to the α - and the β -NH₂-termini of Hb, respectively (10, 20). However, the effects of modifying agents on Hb are not uniquely determined by the locus of their binding to Hb. For example, selective modification of oxy-Hb at the α -NH₂-termini with pyridoxal 5'-sulfate caused only a small reduction in P_{50} , since the sulfate was able to form a physiologic salt bridge with the opposite α globin chain, thereby stabilizing the deoxy conformation (16). Furthermore, this compound primarily inhibited polymerization of Hb S by an oxygen-independent mechanism (3).

The major effect of oral cyanate on *in vivo* sickling was due to the increased oxygen affinity of the erythrocytes (21). The resulting impairment of oxygen transport by these cells stimulated entry of untreated erythrocytes into the circulation and raised the hematocrit, both of which antagonized the benefits of treatment. The use of altered conditions for modification of erythrocytes with cyanate *in vitro* was unsuccessful in preventing the previously observed reduction in P_{50} even though the proportion of cyanate attached to the β -globin chain of Hb was increased (10). In contrast, PLP reduced sickling of erythrocytes without an adverse effect on the oxygen affinity of these cells. PLP might therefore prove more effective as an antisickling agent *in vivo* than either pyridoxal or cyanate.

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