Inhibition of the Pentose Phosphate Shunt by Lead: A Potential Mechanism for Hemolysis in Lead Poisoning

By Neil A. Lachant, Akio Tomoda, and Kouichi R. Tanaka

Recent investigations have disclosed a decrease in pentose phosphate shunt activity in hereditary pyrimidine 5'-nucleotidase deficiency. Clinical lead poisoning is associated with an acquired decrease in pyrimidine 5'-nucleotidase activity. The current investigations were undertaken (1) to determine if pentose shunt activity was decreased in erythrocytes exposed to lead, and (2) to compare the mechanism of inhibition to that seen in hereditary pyrimidine 5'-nucleotidase deficiency. Normal erythrocytes incubated with lead acetate in vitro demonstrated increased Heinz body formation, decreased reduced glutathione, a positive ascorbate cyanide test, and a reversible suppression of pentose shunt activity in the intact erythrocyte. Lead acetate added to normal red cell defense system after lead exposure, and (2) to determine if the mechanism of pentose phosphate shunt inhibition in red cells exposed to lead acetate in vitro was similar to that seen in hereditary erythrocyte pyrimidine 5'-nucleotidase deficiency.

MANY MECHANISMS have been indicted for the development of anemia in clinical lead poisoning.1,2 Most studies have centered around the impairment of heme1,3 and globin synthesis. Despite problems with the 51Cr labeling technique, red cell survival has been shown to be shortened.8,9 Altered intracellular red cell pentose phosphate shunt and antioxidant defense are implicated in the shortened red cell survival.10,11 Increased oxidant sensitivity secondary to inhibition of glutathione (GSH) synthesis has also been suggested as possible mechanisms.1,10,12 Paglia et al. first demonstrated that pyrimidine 5'-nucleotidase activity was decreased in the erythrocyte after exposure to lead in vivo and in vitro.13,14 Recent investigations from this laboratory have suggested that the shortened red cell survival in hereditary pyrimidine 5'-nucleotidase deficiency is due, in part, to suppression of pentose phosphate shunt (PPS) activity caused by the inhibitory effects of increased pyrimidine 5'-nucleotides on glucose-6-phosphate dehydrogenase (G6PD) activity.15,16 The presence of an unexplained hemolytic anemia and impaired pyrimidine 5'-nucleotidase activity in lead poisoning prompted the present investigations to (1) determine the status of the red cell pentose phosphate shunt and antioxidant defense system after lead exposure, and (2) to determine if the mechanism of pentose phosphate shunt inhibition in red cells exposed to lead acetate in vitro was similar to that seen in hereditary erythrocyte pyrimidine 5'-nucleotidase deficiency.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except for tert-butyl-hydroperoxide (ICN Pharmaceuticals, Inc., Plainview, NY) and 14C-glucose (New England Nuclear, Boston, MA). Solutions of sodium acetate and lead acetate were made fresh daily.

Preparation Methods

After obtaining informed consent, venous blood samples were obtained from normal volunteers by routine venipuncture. Heparin (15 U/ml whole blood) was used as the anticoagulant. Blood samples were kept in ice water until used.

General Methods

White cell and platelet-free red cell suspensions were prepared using α-cellulose and microcrystalline cellulose by the method of Beutler.17 Final red cell counts were adjusted to between 2.8 and 3.2 × 10^6/μl.

Unless otherwise stated, a 20% suspension of intact red cells was incubated in a solution of 0.9% sodium chloride and 1 μM glucose, with either 50 μM sodium chloride, 50 μM sodium acetate, or 50 μM lead acetate at 37°C for 30 min. Red cells were then twice washed in normal saline prior to the performance of the studies listed below.

The methods used in this laboratory for determining glycolytic and glutathione peroxidase activities have been previously published.18 Incubated Heinz body formation was determined using an approximately 40% red cell suspension by the method of Beutler,17 except that the buffer contained 30 mg/dl glucose. Reduced glutathione (GSH) stability was determined in conjunction with the Heinz body assay after a 2-hr incubation with acetylphenylhydrazine. The percent stability was calculated by the ratio of the postincubation reduced glutathione (GSH) to preincubation GSH. The ascorbate cyanide test

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Supported in part by Grant AM-14898 from the National Institutes of Health.


Submitted April 1, 1983; accepted September 13, 1983.

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0006-4971/84/0303-0004$0.00/0

was performed by the method of Jacob and Jandi,20 Glycolytic intermediates,21 adenosine triphosphate (ATP) (Sigma Kit 377-UV), and 2,3-diphosphoglycerate (Sigma Kit 35-UV) were measured on perchloric acid extracts by standard methods. Acid extracts of washed red cells were used for the determination of pyrimidine 5'-nucleotide ultraviolet absorption spectra.22 Hemoglobin electrophoresis was done on cellulose acetate at pH 8.6 by standard methods.23

Pentose Phosphate Shunt Activity

Pentose phosphate shunt activity was determined in the preincubated erythrocytes (see General Methods section) by a modification of the method of Davidson and Tanaka.24 The release of \(^{14}\)CO\(_2\) from \(^{14}\)C-1-glucose or \(^{14}\)C-2-glucose was measured in a vibrating reed electrometer and ionization chamber. The original method has been modified so that the build-up of \(^{14}\)CO\(_2\) is measured in a closed system. Fifty microliters of packed red cells was suspended in 1 ml of pH 7.4 Krebs-Ringer bicarbonate buffer. After 1 hr, the system was stimulated with 10 \(\mu\)l of \(10^{-4}\) M new methylene blue (Sigma B-4631). \(^{14}\)CO\(_2\) production was continuously monitored for a total of 2 hr.

Pentose phosphate shunt activity was determined in hemolysates made from an aliquot of the same preincubated red cells by a modification of the method of Smith.25 Krebs-Ringer bicarbonate buffer was supplemented with 1 mM ATP and 2 mM NADP. Fifty microliters of red cell hemolysate (1.5 dilution of packed red cells with distilled water) was added to 400 \(\mu\)l of buffer. New methylene blue was not added to this system.

Enzyme Studies

The direct effect of lead on the activity of red cell enzymes was determined in red cell hemolysates. Assays for the activity of G6PD, 6PGD, glutathione reductase, glutathione peroxidase, transaldolase, transketolase, phosphofructokinase, phosphoglucose isomerase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase were determined in red cell hemolysates. Assays for the activity of the enzyme G6PD were performed by the method of iacob and iandl.26 Pentose phosphate shunt activity was determined in hemolysates from a 1 ml dilution of packed red cells by a modification of the method of Smith.25 Krebs-Ringer bicarbonate buffer was supplemented with 1 mM ATP and 2 mM NADP. Fifty microliters of red cell hemolysate (1.5 dilution of packed red cells with distilled water) was added to 400 \(\mu\)l of buffer.

Statistical Analysis

Two-tailed and paired Student’s t tests were performed by standard methods.26 All data are expressed as mean ± 1 standard deviation. The effect of lead on red cell G6PD activity was determined from Eadie-Hofstee plots, and the effect of lead on G6PD kinetics was calculated from Lineweaver-Burk plots.27

RESULTS

Effect of Lead on Red Cell Metabolism

The effects of lead on red cell metabolic variables are shown in Table 1. Minimal hemolysis occurred when red cells were preincubated with 50 \(\mu\)M lead acetate for 30 min. No hemolysis occurred when red cells were incubated in the sodium chloride or sodium acetate containing solutions. Although Heinz bodies were not present prior to the incubation with acetylphenylhydrazine, erythrocytes preincubated with lead had significantly increased Heinz body formation (\(p < 0.005\)) after incubation with acetylphenylhydrazine. After exposure to lead, the red cell reduced glutathione content was decreased by 11% (\(p < 0.05\)). Although the content of reduced glutathione was decreased in the red cells incubated with lead compared to those incubated with sodium acetate after exposure to acetylphenylhydrazine (341 ± 164 versus 278 ± 171 \(\mu\)g/10\(^{10}\) RBC, \(p < 0.08\)), there was no significant difference in the percent glutathione stability. The ascorbate cyanide test was positive. There were no significant differences in these variables when red cells incubated with sodium chloride were compared to those incubated with sodium acetate, indicating that these metabolic changes were due to lead and not to the presence of acetate.

| Table 1. Metabolic Changes After Incubating Intact Erythrocytes with Lead |
|----------------|------------------|----------------|------------------|-------------------|----------------|
|                | I versus II      | II versus III  | III               |
|                | Value of \(p^*\) | Value of \(p^*\)| Value of \(p^*\)| Value of \(p^*\)| Value of \(p^*\)|
| Heinz body formation (%) | 3 | 13 ± 6 | NS | 13 ± 3 | <0.005 | 82 ± 11 |
| Reduced glutathione \(\mu g/10^{10}\) RBC | 5 | 599 ± 150 | NS | 601 ± 147 | <0.05 | 535 ± 159 |
| Reduced glutathione stability (%) | 5 | 54 ± 15 | NS | 53 ± 19 | NS | 45 ± 22 |
| Ascorbate cyanide test | Neg | Neg | Neg | Neg | Pos | Neg |
| Isopropanol test | Neg | Neg | Neg | Neg | Neg | Neg |

*Paired t test.
NS, not significant.
Effects of Lead on Enzyme Activity, Glycolytic Intermediates, Pyrimidine 5'-Nucleotides, and Hemoglobin in Intact RBC

Intact red cells were preincubated for 30 min with lead, sodium acetate, or sodium chloride and twice washed with saline prior to assay for the activity of the enzymes of the Embden-Meyerhof pathway (hexokinase through lactate dehydrogenase), G6PD, 6PGD, transaldolase, transketolase, glutathione reductase, and glutathione peroxidase. Our findings were in agreement with those of Paglia.13 In the lead-exposed red cells, the largest decrease was in phosphofructokinase activity, which was decreased to 85% of that seen in red cells exposed to sodium acetate. Hexokinase and G6PD activities were basically unchanged, being 95% and 98%, respectively, of that seen in the unleaded red cells; nor were there any significant differences in the intraerythrocytic concentrations of ATP, 2,3-diphosphoglycerate, glucose-6-phosphate, fructose-6-phosphate, or lactate (data not shown). Red cells incubated with lead acetate did not show a shift in the spectral scan for pyrimidine 5'-nucleotides (data not shown).

Red cells incubated with lead did not show hemoglobin instability in the isopropanol test (Table 1). Electrophoresis of hemolysate from the lead-exposed erythrocytes did not show the presence of a fast-moving hemoglobin (data not shown).28

Effect of Lead on Pentose Phosphate Shunt Activity

PPS activity and pentose phosphate recycling through the shunt were measured in intact erythrocytes by the release of $^{14}$CO$_2$ from $^{14}$C-1-glucose and $^{14}$C-2-glucose, respectively, before and after stimulation with new methylene blue. Pentose shunt activity was also measured in hemolysates made from an aliquot of the same batch of red cells. In all studies, erythrocytes were preincubated with the sodium chloride, sodium acetate, or lead acetate solutions and then washed with saline, before measuring the PPS activity.

Pentose phosphate shunt activity was decreased by 45% before ($p < 0.005$) stimulation with new methylene blue and was decreased by 34% after ($p < 0.005$) stimulation, when red cells exposed to lead acetate were compared to red cells exposed to sodium acetate. Stimulated PPS activity was decreased ($p < 0.001$) in sodium acetate exposed red cells when compared to those preincubated with sodium chloride (Table 2). This most likely is a reflection of a decrease in the intraerythrocytic pH due to the presence of acetate.

Conversely, there was no measured difference in the rate of pentose phosphate recycling through the PPS before or after new methylene blue stimulation when red cells incubated with lead acetate were compared to those incubated with sodium acetate. Stimulated pentose phosphate recycling was decreased ($p < 0.05$) in the sodium acetate exposed red cells compared to those incubated with sodium chloride (Table 2), and again, this most likely represents a pH-related effect due to the presence of intraerythrocytic acetate.

Because lead decreased the PPS activity in the intact red cells, PPS activity was measured in red cell hemolysates to determine if lead had caused a reversible inhibition or an irreversible inactivation (denaturation) of the enzymes of the shunt. There were no significant differences in the PPS activities in red cell hemolysates made from red cells exposed to lead acetate, sodium acetate, or sodium chloride (Table 2).

### Effect of Lead on Enzyme Activity in Red Cell Hemolysates

The activities of the pentose phosphate shunt enzymes, glutathione reductase and glutathione peroxidase, and key glycolytic enzymes were determined in fresh hemolysates made from normal red cells. Enzyme activities were determined after incubation of the hemolysate with various concentrations of lead acetate for 15 min (Fig. 1). There was a significant decrease in the activities of G6PD and phosphofructokinase at concentrations of lead achieved in the eryth-

### Table 2. Pentose Phosphate Shunt Activity (µmole Glucose Oxidized · 10$^{10}$ RBC$^{-1}$ · hr$^{-1}$) After Incubating Intact Erythrocytes With Lead

<table>
<thead>
<tr>
<th></th>
<th>I Sodium Chloride</th>
<th>I versus II Value of $p^*$</th>
<th>II Sodium Acetate</th>
<th>II versus III Value of $p^*$</th>
<th>III Lead Acetate</th>
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<tbody>
<tr>
<td><strong>Intact red cell</strong></td>
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<tr>
<td>$^{14}$C-1-glucose</td>
<td></td>
<td></td>
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<tr>
<td>Unstimulated</td>
<td>0.12 ± 0.07</td>
<td>NS</td>
<td>0.11 ± 0.05</td>
<td>&lt;0.005</td>
<td>0.06 ± 0.03</td>
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<tr>
<td>Stimulated†</td>
<td>2.12 ± 0.56</td>
<td>&lt;0.001</td>
<td>1.64 ± 0.44</td>
<td>&lt;0.005</td>
<td>1.08 ± 0.42</td>
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<tr>
<td>$^{14}$C-2-glucose</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Unstimulated</td>
<td>0.08 ± 0.04</td>
<td>NS</td>
<td>0.04 ± 0.01</td>
<td>NS</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Stimulated†</td>
<td>0.97 ± 0.20</td>
<td>&lt;0.05</td>
<td>0.66 ± 0.15</td>
<td>NS</td>
<td>0.72 ± 0.06</td>
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<td><strong>Red cell hemolysate</strong></td>
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<tr>
<td>$^{14}$C-1-glucose</td>
<td>2.42 ± 1.26</td>
<td>NS</td>
<td>1.84 ± 0.92</td>
<td>NS</td>
<td>2.34 ± 0.67</td>
</tr>
</tbody>
</table>

*Paired t test.
† 10 $^\circ$M new methylene blue.
NS, not significant.
The effect of lead on enzyme activity in red cell hemolysate. The reaction mixtures contained 20 μl of normal red cell hemolysate, 0.1 M Tris-HCl buffer, pH 7.1, and appropriate substrates/cofactors. Lead acetate (0–200 μM final concentration) was then added to the reaction mixture. After incubation at 37°C for 15 min, the reaction was started. The residual enzyme activity after lead exposure is expressed as the percent of the enzyme’s activity determined in the absence of lead. The shaded area represents the intraerythrocytic lead concentration attained in clinical leading poisoning.

Phosphofructokinase activity was decreased by 50% with 0.9 μM lead and was completely inhibited by 8 μM lead. G6PD activity was inhibited 50% by 1.8 μM lead, and completely inhibited by 10 μM lead. The activities of 6PGD, glutathione reductase, and hexokinase were less affected, with 6PGD activity being inhibited 50% by 8–9 μM lead. The activities of glutathione peroxidase, transaldolase, transketolase, phosphoglucone isomerase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase were not affected by up to 200 μM lead.

The Effect of Lead on G6PD Kinetics

G6PD activity was measured in hemolysates from normal red cells in 0.1 M Tris-HCl buffer, pH 7.1, with 10 mM MgCl₂. Prior to measuring G6PD activity, hemolysates were preincubated for 15 min with or without lead. G6PD activity was measured in the presence of varying concentrations of NADP and glucose-6-phosphate (G6P). These data are expressed in Figs. 2 and 3 as Eadie-Hofstee plots. Lead was a noncompetitive inhibitor with G6P and NADP for G6PD. When calculated from Lineweaver-Burk plots, the Kᵢ of lead for G6P was 1.5 μM in the presence of MgCl₂ and 4.9 μM in the absence of MgCl₂. The Kᵢ of lead for NADP was 2.1 μM in the presence of MgCl₂ and 4.6 μM in the absence of MgCl₂.

The Effect of Thiol Reagents on the Inhibition of G6PD by Lead

Red cell hemolysates were incubated with various concentrations of 2-mercaptoethanol or reduced glutathione and pyruvate kinase were not affected by up to 200 μM lead.
thione for 15 min at 37°C. They were then incubated for an additional 15 min with lead prior to assay for G6PD activity. Preincubation of red cell hemolysates with reduced glutathione and 2-mercaptoethanol prevented the inhibition of G6PD activity by lead (Fig. 4). The loss of G6PD activity was completely prevented by 0.4–1.7 mM reduced glutathione.

In a similar experiment, red cell hemolysates were incubated for 15 min with lead acetate prior to incubation for 15 min with 2-mercaptoethanol or reduced glutathione. The lead-induced loss of G6PD activity could not be reversed by 0.4 mM reduced glutathione or 10 or 100 mM 2-mercaptoethanol (Table 3).

**DISCUSSION**

Paglia et al. and others have demonstrated that the activity of pyrimidine 5'-nucleotidase is decreased in erythrocytes exposed to lead in vivo or in vitro. Recent investigations from this laboratory have suggested that the hemolytic anemia seen in hereditary pyrimidine 5'-nucleotidase deficiency is due, in part, to an increased sensitivity of the erythrocyte to oxidant stress, which results from a reversible inhibitory effect of the pyrimidine 5'-nucleotides on the pentose phosphate shunt. The primary mechanisms of shunt suppression are: (1) competitive inhibition of the pyrimidine 5'-nucleotides with glucose-6-phosphate for G6PD, and (2) noncompetitive inhibition of the pyrimidine 5'-nucleotides with NADP for G6PD. The increased concentrations of the nucleotide phosphates decrease the intracellular pH, which further suppresses G6PD activity.

Although the various components of the pentose phosphate shunt and glycolytic pathway have been examined in looking for evidence of oxidant sensitivity in the erythrocytes of individuals with clinical lead exposure or toxicity, reproducible confirmatory findings have not been found. The observed pattern of glycolytic intermediates suggests that the activity of the Embden-Meyerhof pathway is normal. In the only study evaluating Heinz body formation, Ghelberg observed that children living near a lead smelter had increased Heinz body formation compared to children living in other industrial areas. The intracellular content of reduced glutathione has been highly variable, being increased, decreased, or unchanged. G6PD activity has been examined without respect to cell age or reticulocyte count and has been reported to be increased, decreased, or unchanged. Abnormalities of glutathione reductase and glutathione peroxidase activities have not been reported.

In the present study, we observed that incubated Heinz body formation was increased, reduced glutathione was decreased, and the ascorbate cyanide test was positive in erythrocytes that had been preincubated with lead acetate. As the normal isopropanol test and hemoglobin electrophoresis suggested that the formation of an unstable lead hemoglobin complex did not occur, we evaluated the activity of the pentose phosphate shunt by the release of 14CO2 in intact red cells before and after new methylene blue stimulation and in red cell hemolysates in order to further explain our findings. Pentose phosphate shunt activity was significantly decreased (p < 0.005) both before and after stimulation.
after new methylene blue stimulation in erythrocytes that were previously incubated with lead acetate. Because the pentose shunt activity of the hemolysates from these same red cells was normal, a reversible inhibition of the pentose shunt is the most likely explanation for the inability of erythrocytes exposed to lead acetate in vitro to handle oxidant stress.

Whereas the release of $^{14}CO_2$ from $^{14}$C-1-glucose was decreased in lead-exposed red cells, the release of $^{14}CO_2$ from $^{14}$C-2-glucose was normal. Although the mechanisms controlling pentose phosphate recycling are poorly understood, the apparently normal pentose phosphate recycling that we observed is most likely a reflection of the effects of other metabolic defects on the flux of glycolytic intermediates and pentose phosphate sugars. The pattern of decreased $^{14}CO_2$ release from $^{14}$C-1-glucose and normal $^{14}CO_2$ release from $^{14}$C-2-glucose is rather unusual, as a relative increase in pentose phosphate recycling is usually seen in conjunction with increased pentose phosphate shunt activity. Ribose-5-phosphate generated by the pentose phosphate shunt may be used as a substrate for the generation of fructose-6-phosphate and glyceraldehyde-3-phosphate or it may be converted to phosphoribosyl pyrophosphate (PRPP) by PRPP kinase. As Paglia has shown that PRPP kinase activity is decreased in red cells exposed to lead in vitro, there should be an increased conversion of $^{14}$C-ribose-5-phosphate and $^{14}$C-ribose-5-phosphate to $^{14}$C-fructose-6-phosphate and $^{14}$C-glucose-6-phosphate due to the metabolic block of the salvage pathway. The increased $^{14}$C-glucose-6-phosphate/glucose-6-phosphate ratio will lead to an increase in the number of molecules of $^{14}$C-glucose-6-phosphate oxidized to $^{14}CO_2$, albeit at a decreased rate. Because the specific activity of the $^{14}$C-glucose-6-phosphate pool cannot be instantaneously determined, a spuriously high rate of pentose phosphate recycling will be calculated.

Lead appears to decrease pentose phosphate shunt activity by inhibiting the activity of G6PD. When lead was added to normal red cell hemolysates, it acted as a noncompetitive inhibitor of both G6P and NADP for G6PD. The calculated $K_i$ of lead for G6P was 1.5 $\mu$M and that for NADP was 2.1 $\mu$M. The intracellular concentration of G6P is about 27 $\mu$M. Its $K_m$ for G6PD is 41.3 $\mu$M in the current study, and 44 $\pm$ 4 $\mu$M at pH 7.3 in the literature. Similarly, the estimated red cell NADP concentration is about 1 $\mu$M with $K_{ms}$ of 34.5 $\mu$M in the current study and 12.5 $\pm$ 2.1 $\mu$M at pH 7.3 in the literature. Because the intracellular lead concentration is approximately 3–6 $\mu$M in clinical lead poisoning, these data strongly suggest that inhibition of G6PD by lead should occur in the red cell in vivo.

It is of interest that magnesium is able to potentiate the inhibitory effect of lead on G6PD. The red cell is rich in magnesium, with an intraerythrocytic concentration of approximately 3 mM. Although red cell magnesium levels have not been evaluated in clinical lead poisoning, changes in the concentration of this cation could have significant effects on enzyme function and red cell survival in the clinical setting.

As the most likely mechanism for the interaction between lead and G6PD is through the formation of a lead–sulfhydryl complex, we evaluated the ability of thiol reagents to protect G6PD from the effects of lead. Thiol reagents were able to prevent the loss of G6PD activity if added to red cell hemolysates prior to the addition of lead. Having a readily available source of reducing equivalents may be an important in vivo protective mechanism for the intact erythrocyte against lead, as subphysiologic (0.4–2.0 mM) concentrations of reduced glutathione protected G6PD against the effects of 2 $\mu$M lead in vitro (Fig. 4). Although thiol reagents can prevent the loss of G6PD activity due to lead, they are unable to reverse the G6PD inhibition if added to red cell hemolysates after lead. It is quite possible that lead acts as a reversible inhibitor of G6PD activity in the red cell cytosol, but would, in addition, inactivate (denature) G6PD in an assay system where enzyme and reduced glutathione concentrations had been diluted 250-fold.

In summary, a reversible suppression of pentose phosphate shunt function due to a decrease in the activity of G6PD occurs in erythrocytes preincubated with lead. Kinetic studies suggest that lead is a noncompetitive inhibitor of both G6P and NADP for G6PD. The manner in which lead inhibits G6PD activity is similar to that of the pyrimidine 5'-nucleotides for NADP, but is different for G6P. Thus, both lead itself and the increased concentrations of the pyrimidine 5'-nucleotides inhibit pentose phosphate shunt function, which should, in turn, render the lead-intoxicated erythrocyte more susceptible to oxidant damage and shortened survival in vivo.

ACKNOWLEDGMENT

The authors wish to thank Dr. Warren Davidson for the use of his ionization chamber and vibrating reed electrometer apparatus; Eileen Kawaguchi Purcell and Eicnin Chang Lim for technical assistance; and Evelyn Larson for secretarial assistance.

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