

Erythrocyte Nucleotides in Children—Increased Blood Lead and Cytidine Triphosphate

CAROL R. ANGLE,^(20, 26) MATILDA S. McINTIRE, MARK S. SWANSON, AND SIDNEY J. STOHS

Departments of Pediatrics and of Biomedical Chemistry of the University of Nebraska Medical Center, Omaha, NE, USA

Summary

The erythrocyte nucleotides of 25 children, 1-5 years old, with normal and increased blood leads, were assayed by anion-exchange high-performance liquid chromatography. Red blood cells of the 12 children with blood lead (PbB) below 30 $\mu\text{g}/\text{dl}$ ($20.3 \pm 6 \mu\text{g}/\text{dl}$, $\bar{X} \pm \text{S.D.}$) had normal levels of activity of pyrimidine 5'-nucleotidase (P5N) and their erythrocytes were virtually free of pyrimidine nucleotides except for small amounts of UMP and UDP. The purine nucleotides, predominantly ATP and GTP, were present at values similar to adults. In the 13 children with PbB 30-72 $\mu\text{g}/\text{dl}$ ($45.3 \pm 14.3 \mu\text{g}/\text{dl}$), total cytidine phosphates (CMP, CDP, CTP) were significantly ($P < 0.001$) increased from trace values to $8.31 \pm 6.21 \text{ nmoles}/10^{10}$ erythrocytes. The purine nucleotides were unchanged. P5N activity was 143.3 ± 22.0 units/g hemoglobin in children with PbB $< 30 \mu\text{g}/\text{dl}$ and 75.4 ± 24.2 units ($P < 0.001$) in the high lead subjects. There was a logarithmic correlation of erythrocyte cytidine phosphates with PbB ($r = 0.89$, $P < 0.001$) and an inverse correlation of cytidine phosphates with \ln P5N activity ($r = 0.59$, $P < 0.001$), of \ln P5N with PbB ($r = 0.64$, $P < 0.001$) and of \ln P5N with \ln erythrocyte zinc protoporphyrin (Protoporphyrin IX) ($r = 0.57$, $P < 0.001$).

Speculation

The accumulation of small amounts of erythrocyte CTP in children with increased lead exposure but with blood concentrations of leads as low as 30 $\mu\text{g}/\text{dl}$ supports a lower threshold for the consequences of lead induced inhibition of pyrimidine 5'-nucleotidase (P5N) than indicated by earlier, less sensitive assays of nucleotides in adults with lead poisoning. The presence of CTP as the predominant pyrimidine nucleotide is similar to the profile in congenital deficiency of P5N but the nucleotides accumulate with less suppression of P5N than found in congenital deficiency. The significance of increased red cell cytidine phosphates at low levels of lead exposure is unknown but it appears to relate to suppression of P5N activity early in red cell maturation, and thus provides an index of antecedant lead exposure that correlates with erythrocyte zinc protoporphyrin.

It is generally accepted that the human erythrocyte is virtually free of pyrimidine nucleotides and is characterized by a selective, possibly unique, enzyme system that favors conservation of the purine nucleotides which serve as the precursors of ATP (25). It was Valentine *et al.*'s (23) description of a congenital hemolytic anemia characterized by the intraerythrocyte accumulation of pyrimidine nucleotides that led to the definition of a pyrimidine 5'-nucleotidase (P5N) (E.C. 3.1.3.5) in the erythrocyte cytosol. P5N specifically dephosphorylates the pyrimidine nucleoside monophosphates OMP, UMP, and CMP, allowing diffusion from the cell as the soluble nucleosides: cytidine and uridine (14).

Congenital deficiency of P5N, typically with less than 10% of normal enzyme activity, is relatively frequent among the definable causes of nonspherocytic, familial hemolytic anemia (11, 12, 23).

It may be manifested by early and recurrent jaundice, has a possibly increased association with mental retardation (6) and is characterized by a basophilic stippling of the erythrocytes resembling lead poisoning. Of analogous interest, P5N is readily inhibited by lead both *in vitro* and *in vivo* (14). Clinical studies by ourselves (2, 3, 9, 11) and others (8, 11, 16) have established the logarithmic suppression of erythrocyte P5N evident at even low levels of blood lead, but the effect of this partial inhibition of P5N on the erythrocyte nucleotides of children has not previously been reported. This is also the first report, employing high performance liquid chromatography technology, of the normal nucleotide profile of young children.

In this study the erythrocyte nucleotides of 25 children, 1-5 years old, were assayed by anion-exchange high performance liquid chromatography to define the values in control subjects and in those previously identified by the Omaha Lead Paint Poison Prevention Program as having an excessive lead burden. At the time of the testing, 13 children had blood leads in the range of 30-72 $\mu\text{g}/\text{dl}$ but none had anemia or reticulocytosis. Assays in these 25 children and 22 others were utilized to define the intercorrelations of blood lead, cytidine phosphates, P5N, and erythrocyte protoporphyrin IX (zinc protoporphyrin or ZPP).

MATERIALS AND METHODS

The participants in the study were 47 children 1-5 years old followed in the Omaha Lead Paint Poison Prevention Program because of a previous blood lead (PbB) over 30 $\mu\text{g}/\text{dl}$ or erythrocyte zinc protoporphyrin (ZPP) over 60 $\mu\text{g}/\text{dl}$ and 22 healthy children of the same age range seen in our outpatient clinic. Not all blood samples were adequate for all assays and the distribution of the number of subjects for each of the various combinations of assays was as follows:

Assay	Lead Project	Out-Patient	Total
ZPP, P5N	25	22	47
PbB, P5N	20	22	42
PbB, Nucleotides	19	6	25
PbB, P5N, Nucleotides	7	5	12

Because of the significant correlations of data with the standard indices of lead absorption, PbB and ZPP, the analyses according to the two classes of subjects are not incorporated in the data presentation. Informed parental consent was received for all studies. The study was annually approved by the peer professional review committee of the University of Nebraska Medical Center.

Before nucleotide assay the red cells were first freed of platelets and white cells by elution with 5 volumes of saline from 15 ml syringes packed with equal parts of microcrystalline cellulose and alpha cellulose at 4°C (5, 19). All assays were performed on venous blood.

Nucleotides were extracted from the erythrocytes by the method of Chen, *et al.* (9), which involves erythrocyte hemolysis in distilled water, protein precipitation with 10% trichloroacetic acid, and

neutralization of the trichloroacetic acid with tri-N-octylamine in Freon 113 (Matheson Gas Products, Inc.) after centrifugation. The aqueous extracts were immediately frozen at -17°C . Samples were analysed within 5 days after extraction but found to be stable at this temperature for several wk. The extraction efficiency was calculated to be 85–100% for all nucleotides.

The chromatographic system for nucleotide analysis consisted of two model 110 A pumps and a model 420 microprocessor from Altex Scientific, Inc., Berkeley, CA; a model 1203 fixed wavelength UV detector operating at 280 nm from Laboratory Data Control, River Beach, FL; and a variable volume sample injector from Rheodyne, Inc., Berkeley, CA. Chromatographic columns were 25×0.46 cm Partisil 10 SAX anion exchange columns from Altex Scientific, Inc., Berkeley, CA.

Nucleotide separation was accomplished by gradient elution with a low concentration eluent of 0.007 M KH_2PO_4 at pH 3.90 and a high concentration eluent of 0.25 M KH_2PO_4 and 0.50 M KCl at pH 4.50. An isocratic period of 6 min followed by a series of short linear gradient steps forming a 35 min concave gradient from 0–100% enabled complete 5'-nucleotide separation in less than 70 min. The column was then re-equilibrated with 45 ml of the low concentration eluent. The flow rate was constant at 1.5 ml/min and the column was at ambient temperature.

Chromatographic peaks were identified by comparison with reference standards from Sigma Chemical Co., St. Louis, MO. Well resolved separations were obtained of the following 5'-nucleotides: the monophosphate ribonucleosides of cytidine (CMP), uridine (UMP), adenosine (AMP), guanosine (GMP), inosine (IMP) and xanthosine (XMP); the di- and triphosphate ribonucleosides of cytidine (CDP and CTP), uridine (UDP and UTP), adenosine (ADP and ATP) guanosine (GDP and GTP), and inosine (IDP and ITP). Quantitation of the nucleotides was accomplished by relative peak areas of the known standards to the internal standard XMP.

P5N activity of erythrocytes was assayed by the method of Torrance, *et al.* (21). One unit is defined as the enzyme activity that will hydrolyze 1 nmole ^{14}C MP/min/g hemoglobin. Whole blood lead was measured by atomic absorption spectroscopy by the microcup method (10). Although red cell lead is a better index of the lead burden than whole blood lead, particularly in anemic subjects, the decision was made to utilize whole blood lead as the more universal standard of exposure. Erythrocyte zinc protoporphyrin was measured by the method of Lamola (13) employing an ESA Zn P Model 4000 hemofluorometer (Cambridge, MA). One ZPP unit is defined as the presence of 1 g of zinc erythrocyte protoporphyrin/dl whole blood. All values are given as the mean \pm S.D.. Differences in mean values for normal data were analyzed for statistical significance by a one-tailed Student's *t* test with the Mann-Whitney U test used for non-normal data.

RESULTS

The chromatographic separation of the erythrocyte nucleotides identified the 13 major 5'-nucleotides.

Blood samples from the six outpatients, considered to be control subjects had either no cytidine phosphates present (four children) or less than 0.25 nmoles/ 10^{10} red blood cells (rbc) (two subjects). As shown in Figure 1, the presence of measurable amounts of cytidine phosphates increased with the blood lead and cytidine phosphates were found in all 10 samples from children with blood leads of 37–72 $\mu\text{g}/\text{dl}$. Cytidine phosphates were found in 11 of 13 samples from children with a blood lead ≥ 30 $\mu\text{g}/\text{dl}$ but in only four of 12 samples with a blood lead < 30 $\mu\text{g}/\text{dl}$.

Since a blood lead of 30 $\mu\text{g}/\text{dl}$ is the current level considered indicative of excessive lead exposure, the nucleotide profiles were examined for these two groups. (Table 1). The 13 children with a PbB ≥ 30 $\mu\text{g}/\text{dl}$ (30–72 $\mu\text{g}/\text{dl}$) had a mean PbB of 45.3 ± 14.3 , significantly higher than the 20.3 ± 6.0 $\mu\text{g}/\text{dl}$ of the 12 children with a PbB of 8–29.9 $\mu\text{g}/\text{dl}$. The content of the purine nucleotides, of IMP and UDP and UMP were comparable for the two groups and resembled the nucleotide profile of normal adults. The ATP content of 1322.2 ± 279.6 nmoles 10^{10} rbc was similar to the 1204.7

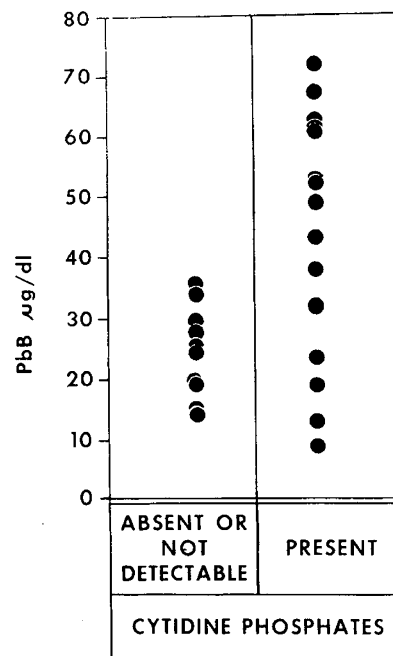


Fig. 1. Of 25 red cell samples, cytidine phosphates were found in 15. All subjects with blood leads > 38 $\mu\text{g}/\text{dl}$ had measurable amounts. No cytidine phosphates were found in the erythrocytes of eight of 12 children 1–5 yr old with a blood lead (PbB) < 30 $\mu\text{g}/\text{dl}$; measurable amounts were found in 11 of 13 samples from children with blood lead ≥ 30 $\mu\text{g}/\text{dl}$.

Table 1. Erythrocyte nucleotides nmoles/ 10^{10} RBC, $\bar{X} \pm$ S.D. Children 1–5 yrs old

Nucleotide	PbB ≥ 30 $\mu\text{g}/\text{dl}$ <i>n</i> = 13	PbB < 30 $\mu\text{g}/\text{dl}$ <i>n</i> = 12
ATP	1285.17 \pm 320.40	1209.17 \pm 341.27
ADP	216.0 \pm 160.02	152.73 \pm 49.45
AMP	56.67 \pm 17.68	30.3 \pm 11.13
GTP	109.15 \pm 49.72	81.96 \pm 35.65
GDP	31.04 \pm 31.72	21.14 \pm 3.59
GMP	2.92 \pm 1.45	15.4 \pm 27.60
IMP	64.5 \pm 54.34	65.96 \pm 38.49
CTP	10.64 \pm 10.65 ¹	0.02 \pm 0.07
CDP	3.88 \pm 5.87	0.00 \pm 0.06
CMP	0.56 \pm 0.84	0.29 \pm 0.50
UTP	1.27 \pm 2.39	0.60 \pm 0.00
UDP	2.44 \pm 2.75	0.27 \pm 0.85
UMP	5.87 \pm 8.67	6.73 \pm 9.96
PbB $\mu\text{g}/\text{dl}$	45.3 \pm 14.3 ¹	20.3 \pm 6.0

¹ $P < 0.001$, Mann-Whitney U test.

± 323.1 of the low lead subjects and to the generally accepted adult value of 1000 nmoles/ 10^{10} . The only difference in the erythrocyte nucleotide profiles between the two groups was the significant ($P < 0.001$, Mann-Whitney U test) increase in the cytidine phosphates, primarily as CTP, in the samples from the 13 children with PbB ≥ 30 $\mu\text{g}/\text{dl}$, in contrast to the trace values of the 12 children with PbB < 30 $\mu\text{g}/\text{dl}$. In the high lead group, CTP was increased to 10.64 ± 10.65 nmoles/ 10^{10} rbc.

Samples from 15 children, 11 of whom had PbB ≥ 30 $\mu\text{g}/\text{dl}$, had measurable amounts of cytidine phosphate. As shown in Figure 2, there was a significant logarithmic correlation of total erythrocyte cytidine phosphate content, in nmoles/ 10^{10} rbc (Y) with PbB $\mu\text{g}/\text{dl}$ (X): $\ln Y = 2.44 \ln X - 7.13$; $r = 0.89$; $P < 0.001$. This regression predicts the accumulation of 329 nmoles of cytidine phosphates at PbB 200 $\mu\text{g}/\text{dl}$.

The correlation of red cell P5N (Y) and blood lead (X) as determined in 42 children (Fig. 3) again established a significant inverse logarithmic correlation: $\ln Y = 5.062 - 0.016X$; ($r = 0.64$; $n = 42$; $P < 0.001$). In 27 children with a PbB < 30 $\mu\text{g}/\text{dl}$, P5N activity was 143.1 ± 22.0 units/g hemoglobin (mean \pm S.D.), which is similar to our laboratory normal of 148 ± 20 . In 15

children with $PbB \geq 30 \mu\text{g/dl}$, P5N (mean \pm S.D.) was 75.4 ± 24.3 or 53% of the low lead group.

As shown in Figure 4, P5N was also inversely correlated with ZPP, the index of lead exposure most commonly employed in community programs. Eight of the nine children with ZPP $>60 \mu\text{g/dl}$ had significant decreases in P5N below the normal range of 128–168 units. The regression equation of the correlation of P5N (Y) and ZPP (X), $\ln Y = 5.3939 - 0.23 \ln X$ ($n = 47$; $r = 0.57$; $P < 0.001$) predicts a decrease of P5N to 45 units as ZPP increases to $1000 \mu\text{g/dl}$.

The correlation of the lead-induced suppression of P5N (X) with accumulation of cytidine phosphates (Y) was investigated in the data from 12 children with measurable amounts of cytidine phosphates: $Y = 59.14 - 11.48 \ln X$; $r = 0.80$; $P < 0.001$ (Fig. 5). This regression equation predicts an increase in total erythrocyte cytidine phosphates to only $41 \text{ nmoles}/10^{10}$ when P5N is reduced to 5 units or to less than 5% of normal.

DISCUSSION

The red cell nucleotides of children are shown in this study to be quantitatively and qualitatively similar to those of adults. Red

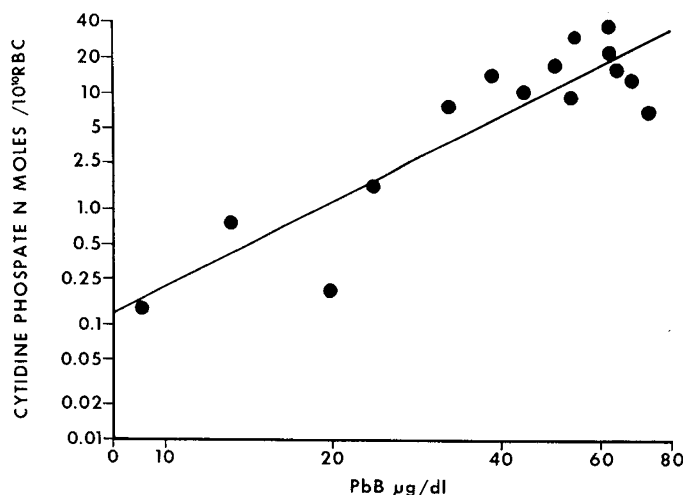


Fig. 2. Total red cell cytidine phosphates in $\text{nmoles}/10^{10}$ rbc (Y) as related to blood lead (PbB; X axis) in the 15 children, 1–5 yr old, with measurable amounts of cytidine nucleotides: $\ln Y = 2.44 \ln X - 7.13$; $r = 0.89$; $P < 0.001$. This regression predicts 329 nmoles of cytidine phosphate at a PbB of $200 \mu\text{g/dl}$.

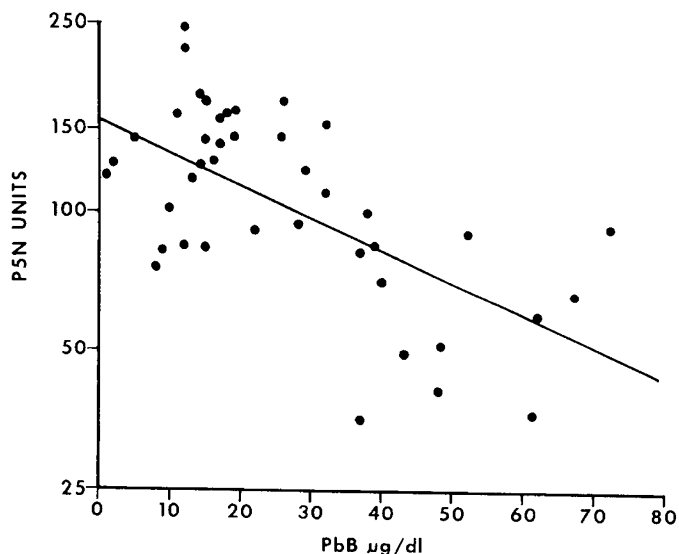


Fig. 3. Correlation of erythrocyte pyrimidine 5'-nucleotidase (P5N; Y) in units defined as the hydrolysis of 1 n mole CMP/min/g hemoglobin with blood lead $\mu\text{g/dl}$ (PbB; X) in 42 children 1–5 yr old: $\ln Y = 5.062 - 0.016 X$; $r = 0.64$; $P < 0.001$. This regression predicts P5N activity of 1.8 units at a PbB of $200 \mu\text{g/dl}$.

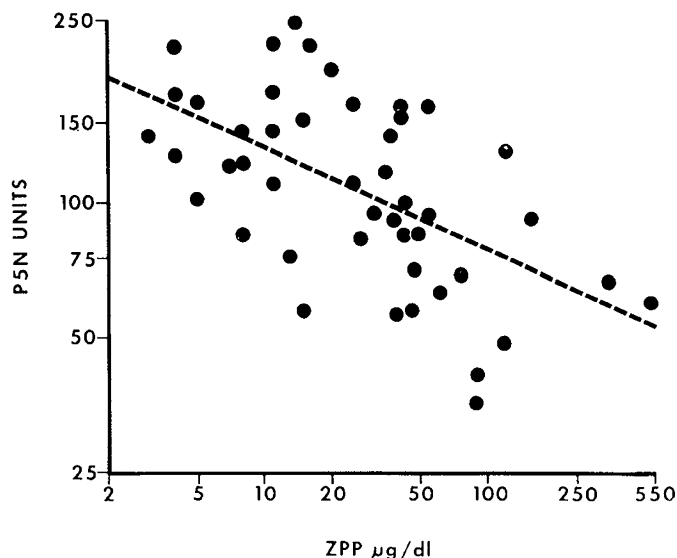


Fig. 4. Correlation of P5N activity (Y) with erythrocyte zinc protoporphyrin $\mu\text{g/dl}$ (ZPP; X) as predicted by data from 47 children: $\ln Y = 5.3929 - 0.23 \ln X$; $r = 0.57$; $P < 0.001$.

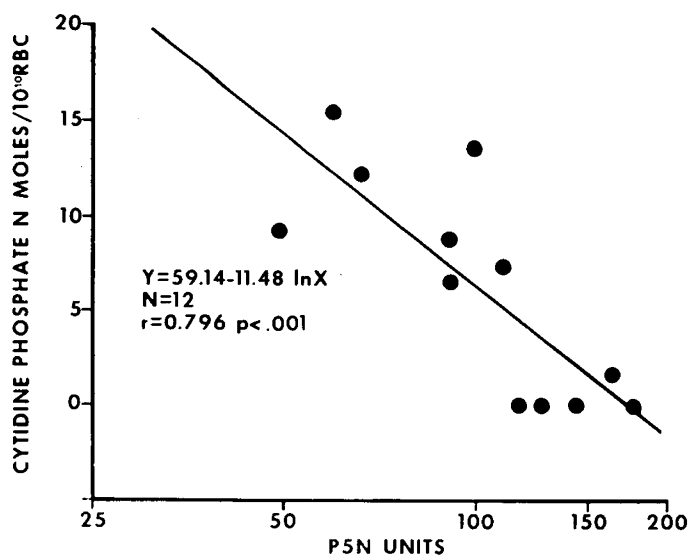


Fig. 5. Correlation of total red cell cytidine phosphates $\text{nmoles}/10^{10}$ rbc (Y) with erythrocyte P5N (X) in 12 children, 1–5 years old: $Y = 59.14 - 11.48 \ln X$; this predicts 41 nmoles cytidine phosphate when P5N activity is reduced to 5 units.

cells are normally free of pyrimidine nucleotides except for small amounts of UDP. In this study of children 1–5 years old, detectable increases in pyrimidine nucleotides, primarily as cytidine triphosphate, correlated with increases in the blood lead and concomitant suppression of the pyrimidine-specific 5'-nucleotidase of the red cell cytosol (P5N).

The sensitivity of the high performance liquid chromatography assay of red cell nucleotides made it possible to show the accumulation of cytidine phosphates at blood leads as low as $30 \mu\text{g/dl}$ and at P5N activity of approximately 30–70% of normal. In congenital deficiency of P5N, spectrophotometric assays have not shown pyrimidine nucleotide accumulation until enzyme activity has been suppressed to below 10%; in adults with severe, symptomatic lead poisoning, pyrimidines were found when P5N was reduced to 15–16% activity (16). The slope of the observed correlation of PbB and P5N (Fig. 2) predicts negligible, or 10%, activity of P5N at a blood lead of $147 \mu\text{g/dl}$ or a red cell lead of $210 \mu\text{g/dl}$. This is comparable to the prediction by Paglia *et al.* (16) of complete suppression of enzyme activity at $200 \mu\text{g/dl}$ of red cell lead. Inasmuch as our previous high performance liquid chro-

matographic assays of red cell nucleotides in lead-poisoned rabbits showed quantitatively similar responses to this childhood study, apparent differences in the sensitivity of adults and children for accumulation of pyrimidine nucleotides by lead-induced suppression of P5N are thought to relate to the method of assay. The effect of lead-induced suppression, however, may be both quantitatively and qualitatively distinct from the sequelae of congenital deficiency of P5N.

The observed correlation of PbB and cytidine phosphates (Fig. 1) predicts an increase in cytidine phosphates to only 329 nmoles/ 10^{10} rbc at a PbB of 200 μ g/dl despite a concurrent prediction of almost complete suppression of enzyme activity. This is far lower than the massive increases of pyrimidine nucleotides to over 1500 nmoles/ 10^{10} rbc in persons found homozygous for deficits of P5N activity (6, 12, 23, 25). This would suggest qualitative differences in the sequelae of the two modes of decreased P5N activity as well as differences that may relate to a sustained suppression of P5N throughout erythrocytic maturation in congenital deficiency *versus* a relatively transient suppression induced by the daily variabilities in blood lead.

The distribution of the erythrocyte pyrimidine nucleotides in children with lead poisoning shows a greater increase in cytidine than uridine phosphates and a predominance of CTP. This is similar to the nucleotide profile noted by Torrance and Whittaker (22) in a child with congenital deficiency of P5N and by our laboratory (19) in studies of lead-treated rabbits. The presence of CTP as the dominant pyrimidine nucleotide is thought to reflect the relative rate of product formation of P5N for OMP $>$ UMP $>$ CMP (15). Continued activity of the diphosphokinases would favor the conversion of CMP \rightarrow CDP \rightarrow CTP but any postulated preference for this over the conversion of UMP \rightarrow UDP \rightarrow UTP requires delineation of the enzyme kinetics in the red cell (1).

Although Boninsegna *et al.* (7) suggest that the soluble fraction of the erythrocyte is unique among blood cells in the activation of UDP \rightarrow UTP by a zinc sensitive enzyme that is distinct from adenylate kinase, there are no comparable studies of erythrocyte activation of CDP \rightarrow CTP. Similarly, additional evidence is required in the mature red cell of the amination of UTP to CTP by CTP synthetase.

In the absence of evidence for a significant *de novo* synthesis of CTP in the mature erythrocyte, Valentine's view of the pyrimidines as residual to RNA of the normoblast is a more tenable hypothesis concerning the origin of the erythrocyte pyrimidine nucleotides. This is also consistent with our previous studies in lead poisoned rabbits (19), in which lead poisoning induced rapid *in vivo* suppression of P5N with a lag of almost 30 days before cytidine phosphates were detected in the red cell. The accumulation of pyrimidine nucleotides and of stippling peaked at 60 days, the time required in the rabbit for a complete turn over of circulating erythrocytes.

The concept of residual RNA as the source of the pyrimidines is also compatible with the basophilic stippling that is characteristic of both lead-induced suppression and congenital deficiency of P5N. Basophilic stippling is generally accepted as an aggregation of ribosomal RNA around the red cell mitochondria (4, 17, 18). The precipitation of unstable ribosomes requires the availability of divalent cations or oxidant activity to effect nucleic acid cross-linkage. Beutler (6) implies that lead poisoning and P5N deficiency may have the common denominator of an excess of divalent cation since the red cell stippling of P5N in both states is reversed by EDTA. It can also be proposed that an excess of pyrimidine nucleotides in the young red cell inhibits ribosomal degradation as well as effecting an abnormal charge that would favor aggregation. The simultaneous inhibition of ribosomal degradation by lead may favor ribosomal instability and precipitation at lesser levels of pyrimidine nucleotide accumulation than found in congenital deficiency.

The presence in red cells of a cytosolic nucleotidase (P5N) that specifically dephosphorylates the pyrimidines, facilitating their diffusion with simultaneous conservation of the purines ATP and

GTP, would suggest the possibility that even low levels of pyrimidine nucleotides may exert a toxic effect. It is agreed that lead-induced nucleotidase deficiency can be sufficiently severe to mimic genetic impairment (25). Although the significance of the accumulation of red cell cytidine phosphates at low levels of lead exposure is unknown, it does appear to require suppression of P5N early in red cell maturation and thus provides an index of cumulative lead exposure during the genesis of the circulating red cells. The mechanism is distinct, but the time lapse is similar to the increase in erythrocyte zinc protoporphyrins due to inhibition of heme synthesis.

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26. Requests for reprints should be addressed to: Carol R. Angle, M.D., Department of Pediatrics, University of Nebraska Medical Center, 42nd and Dewey Avenue, Omaha, Nebraska 68105 (USA).
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