Lead Toxicity and Heme Biosynthesis

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ABSTRACT

Lead intoxication results in a disturbance of heme biosynthesis, its degree depending on the severity and duration of exposure to lead. A mild secondary, sideroblastic anemia is common; basophilic stippling may occur, especially in severe lead poisoning. Increased excretion in the urine of delta-aminolevulinic acid and coproporphyrin III may occur; porphobilinogen excretion is not usually increased. Delta-aminolevulinate dehydratase, coproporphyrin oxidase, and ferrochelatase activities are reduced; delta-aminolevulinate synthetase activity is increased. Erythrocyte protoporphyrin (FEP and ZPP) is increased. Recent knowledge of the heme biosynthetic enzymes is reviewed and the significance of FEP and ZPP discussed. A brief history is given of the relationship of lead toxicity to the porphyrins.

Historical Introduction

Lead has been widely used since ancient times, probably because of its low melting point and ease of working and the abundance of lead-containing minerals and ores. Lead poisoning also has an ancient history. The first association of exposure to lead with abdominal colic and skin pallor (effects of chronic lead poisoning) has been attributed to Hippocrates (ca. 370 B.C.). The victim was a metal extractor. By the first century A.D., the relationship between chronic lead ingestion and abdominal colic, skin pallor and "swelling of the body" was well-known. In medieval times, lead poisoning, known as saturnism (saturn was the alchemists' name for lead) and the occurrence of "pal-sies" was recognized. The first complete clinical description of lead poisoning was given in 1839, and the anemia of lead poisoning was first directly verified by counting the red cells in 1840. Punctate basophilia of the red cells was first recorded in 1899. This observation followed shortly after the introduction of the Romanowsky stains and was considered for many years to be a sensitive and important diagnostic criterion of lead poisoning. It must be appreciated that, until the development of sensitive methods for measuring blood lead, the diagnosis of lead poisoning was based on a history of chronic exposure to lead and the characteristic signs and symptoms, including the
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Lead blue line of the gums, rarely seen today. Punctate basophilia is frequently found in florid cases of lead poisoning.5

Occurrence of red colored urine in some cases of lead poisoning was an old observation. The color was attributed to blood. Stokvis54 (1899) appears to be the first to have identified "hematoporphyrin" in the urine and feces of a patient suffering from severe lead poisoning.5 Hematoporphyrin, produced by treating hemoglobin with strong sulphuric acid, is now known not to occur in urine in health or disease. The pigment described by Stokvis and many later workers were probably a mixture of coproporphyrin and uroporphyrin. Teleky56 (1919) described hematoporphyrinuria as an early diagnostic sign of lead poisoning, but the recognition of the true nature of the urinary pigment was not possible until the chemical structure of the porphyrins had been worked out by Kuster24 (1920). Grotepass25 (1925) showed that the urinary porphyrin was coproporphyrin III, identical with the naturally occurring coproporphyrins of blood. Van den Bergh and Grotepass (1933)59 described patients with lead poisoning, who had an increase of red cell porphyrin, type III, without porphyrinuria. They recommended measurement of red cell porphyrins in the investigation of lead poisoning. The technical difficulty of this measurement restricted to its use to specialized centers. The synthesis of the porphyrins by Fischer over a period of 20 years (described in his book of 1937)19 marked the beginning of the modern era of the study of porphyrin chemistry. Application of this knowledge to the investigation of disease was made possible by the development of new analytical techniques.15,24,44,62

The biochemistry of the porphyrias was investigated in many centers, and some of the results were applied to the diagnosis of lead poisoning. Methods, although still complex, were simplified for clinical use, and free erythrocyte protoporphyrin measurements became possible. Watson and co-workers60 demonstrated an increase in FEP in children with lead poisoning. A great advance in the study of lead poisoning in children was made possible by the introduction by Piomelli45 (1973) of a simple, rapid, micromethod for free erythrocyte protoporphyrin. It measured coproporphyrin as well, but fortunately (for the analyst) this porphyrin is at the most only slightly increased in lead poisoning, whereas free erythrocyte protoporphyrin is often markedly increased. Other micromethods followed,16,48 but the next (and most recent) major advance was the discovery by Lamola and co-workers (1974) that free erythrocyte protoporphyrin was essentially zinc protoporphyrin.53 A new instrument, the Hematofluorometer,9,10 was designed and is now commercially available.* Zinc protoporphyrin concentration can be measured by its use in a drop of blood, without prior chemical treatment.

While the chemistry of the porphyrins was being elucidated, the biochemical synthetic pathway was also being investigated. Progress was rapid after isotopes (both radioactive and non-radioactive) had become available. By 1954, the essential features of heme biosynthesis were known50 and the in vitro effects of lead on the enzymes had been investigated. However, the difficulty of assaying the enzymes has prevented their widespread application to the investigation of lead poisoning. In fact, only one enzyme—delta-aminolevulinate dehydratase or ALA-D—has been extensively investigated and has been shown to decrease in activity as blood lead rises.22 The difficulties of measurement arise because the enzymes must be measured in red cell hemolysates or leucocyte extracts. Most of

* ESA model 4000, (Environmental Science Associates, Bedford, MA 01730); Aviv (Aviv Associates, Lakewood, NJ 08701); and Bromberg (Dr. Bromberg, Weston MA 02193).
the enzymes are labile and must be reactivated. Irreversible losses of enzyme activity may occur during the extraction procedures, and lead contamination may occur during these and the analytical stages.

Since 1950, a new porphyrin in the biosynthetic pathway has been discovered (harderoporphyrin, 1974)\textsuperscript{32} and a previously postulated enzyme has been shown to exist (protoporphyrinogen dehydrogenase\textsuperscript{29,30}). In spite of the vast amount of work on lead poisoning, little is known about the mechanism of lead toxicity, apart from its effects on heme biosynthesis in the bone marrow and depression of globin synthesis\textsuperscript{23,24,47}. Currently, whole blood lead and zinc (or free) erythrocyte protoporphyrin are measured as indicators of lead toxicity, but they may very well not be the most appropriate ones.

**Biosynthesis of Heme**

To follow the various steps in the biosynthesis of heme, a brief account of the structure of the porphyrins will be given. The parent compound, porphyrin, (figure 1) is an essentially planar compound made of four pyrrole units linked by CH (methene) groups. The pyrrole rings are lettered A,B,C,D. Side chains are attached, two in each ring, in positions 2,3; 7,8; 12,13; and 17,18. In the porphyrin compounds, there are only two hydrogen atoms attached to the four nitrogen atoms; in the porphyrinogen compounds, each nitrogen has a hydrogen atom and the methene bridges are reduced to methylene (CH\textsubscript{2}). The porphyrinogens are converted to the porphyrins by non-enzymatic oxidation except for protoporphyrin IX. The side-chains are acetic (\(-\text{CH}_2\text{COOH}\) or A), propionic (\(-\text{CH}_3\text{CH}_2\text{COOH}\) or P), vinyl (\(-\text{CH} = \text{CH}_2\) or V) and methyl (\(-\text{CH}_3\) or M). The side chains of the porphyrins involved in heme synthesis are given in table I. It will be seen that they all have P in positions 13 and 17. The naturally occurring porphyrin in this pathway are type III, i.e., the side chains or rings A and B are in an inverted order, e.g., A,P : P,A. In the I series, they are in cyclic order, e.g., A,P : A,P. The final porphyrin in the series is protoporphyrin IX, the number referring to one of the stereoisomers with the same molecular formula. It is still a type III porphyrin. Heme formed by insertion of a ferrous (Fe\textsuperscript{2+}) atom into protoporphyrin IX, is properly called heme b (figure 2); heme a and heme c, in which different side chains occur in rings A and B, are found in some of the cytochromes, which are also heme proteins.

The main stages in the biosynthesis of heme are the formation of a pyrrole, combination of four pyroles to form a porphyrin,

![Figure 1](image_url)

**Figure.** 1. (A) The parent porphyrin structure, with modern numbering of the atoms. (B) Heme b. (C) Porphobilinogen. The carbons at 5,10,15,20 are methene (CH\textsubscript{2}) in the porphyrins and methylene (CH\textsubscript{2}) in the porphyrinogens.
rin, conversion of the acetic acid side chains in rings A and B to methyl, and then conversion of the acetic groups in rings C and D to methyl, to form protoporphyrinogen IX. This is oxidized to protoporphyrin IX, iron is inserted, and heme results. Every step is the result of the action of one or more enzymes, which are situated in the mitochondrion or cytosol. Heme synthesis can therefore only occur in cells containing mitochondria; in particular, it cannot occur in the mature erythrocyte. Most cells synthesize heme, but only erythrocyte precursors can synthesize the globins required for the formation of hemoglobin. Other cells synthesize appropriate apoproteins for the formation of other heme proteins. The outline of heme synthesis is given in figure 3, which also indicates the location of the enzymes. The essential features of the synthesis have been confirmed in the human by study of patients with porphyrias owing to enzyme deficiencies.

STAGE ONE

The first stage of the biosynthesis of heme is the condensation of glycine and succinyl-coenzyme A to yield delta-aminolevulinic acid (δALA), NH₂CH₂COCH₂CH₂COOH. Succinyl-CoA is a readily available substrate formed from succinate and coenzyme A by the enzymatic action of succinyl-CoA synthetase, with the aid of nucleosidetriphosphate (NTP) and magnesium ions. It is also formed from methylmalonyl-coenzyme A through the action of methylmalonyl isomerase and coenzyme-B₁₂ (in vitamin B₁₂ deficiency in man, methylmalonic acid excretion is increased). The condensation of glycine and succinyl-CoA is catalyzed by 5-aminolevulinate synthetase (ALA-S). Pyridoxal phosphate obligatory for the reaction.⁴²

\[
\text{NH}_2\text{CH}_2\text{COOH} + \text{HOOC.CH}_2\text{CH}_2\text{CO(SCoA)} \rightarrow \text{NH}_2\text{CH}_2\text{COCH}_2\text{CH}_2\text{COOH} + \text{CoASH} + \text{CO}_2
\]

In this reaction, pyridoxal phosphate, which is bound to the enzyme, first combines with glycine to form a Schiff base (combination of the –CHO of pyridoxal and the NH₂ – of glycine to form –CH=N –). As a result, the usually stable glycine becomes highly reactive, forms a carbamation (i.e., a carbon atom in the compound bears a negative charge), and combines with succinyl-CoA.¹

ALA-S is the rate-limiting enzyme of heme synthesis.⁵⁰,⁵² It is an inducible mitochondrial enzyme normally present in low concentration. Its production is stimulated when porphyrins accumulate for various reasons, and it is inhibited by the end product, heme (negative feedback). Although ALA-S acts in the mitochondrion, the enzyme is synthesized in the cytoplasm.²⁶ It can also be induced by hypoxia⁶⁰ and erythropoietin.¹¹

STAGE TWO

The second stage involves the condensation of two molecules of δ-ALA to form porphobilinogen, the structural unit of the porphyrins, with the elimination of two molecules of water (figure 3). The cytosolic enzyme, δ-aminolevulinate dehydratase(ALA-D), catalyzes the reaction. The mechanism of this remarkable condensation has been extensively studied, but has been only partially elucidated.⁵¹ ALA-D has a molecular mass of 280,000 and consists of eight identical subunits, each of molecular mass 35,000 occupying the corners of a cube. Activity of the enzymes requires SH groups. The enzyme also contains about four to six Zn²⁺ atoms per molecule. The enzyme combines with its substrate δ-ALA forming a Schiff base through the ε-amino group of lysine. Only four of the eight subunits form Schiff bases. Another aminoacid, probably histidine⁵⁸ is also involved in the enzymatic reaction.

It has been proposed⁸ that a functional dimer (two subunits of ALA-D) is formed
by several interactions, e.g., ionic and hydrogen-bonding, and covalent linkage of SH groups with Zn$^{2+}$. Two molecules of δ-ALA are sandwiched between the subunits; one molecule of δ-ALA forms a Schiff base between its carbonyl (CO) group and the ε-NH$_2$ of lysine. Histidine, which forms part of the active center, transports a proton from the CH$_2$ next to the CO, creating a reactive carbanion (C$^-$H). A series of reactions then takes place, resulting in the combination of two molecules of δ-ALA and the formation of porphobilinogen. ALA-D is deactivated by oxidation of its SH groups. Activity may be restored in vitro by reduction with sulphydryl reducing agents, such as glutathione, dithiothreitol and mercaptoethanol. Activation by zinc ions occurs only when the enzyme is in its reduced state. It is probable that in vivo the enzyme is maintained in its reduced state by glutathione reductase$^{14}$ and possibly other enzyme systems.

**STAGE THREE**

The third stage of the biosynthesis, the enzymatic polymerization of four molecules of porphobilinogen (PBG) to form uroporphyrinogen (UPG) takes place in the cytosol.$^{20}$ Ammonia is produced; hence a deaminase is involved. UPG (table I) is a cyclic tetrapyrrole occurring in the body in two isomeric forms, UPG I and UPG III. The former occurs in trace amounts in normal subjects but is increased in erythropoietic porphyria, in which disease there is a deficiency of uroporphyrinogen III cosynthetase. UPG III is the parent of the naturally occurring heme proteins in man. Conversion of PBG to UPG III in the cytosol is affected by two enzymes acting together (not in sequence), porphobilinogen deaminase and uroporphyrinogen III cosynthetase. The enzyme pair is sometimes called porphobilinogenase$^7$ and can be assayed as such. UPG I is formed by a sequential head to tail condensation of four molecules of PBG and the elimination of four molecules of ammonia. The resulting tetrapyrrole has eight side chains in the cyclic order A,P,A,P,A,P,A,P. In this condensation, the CH$_2$ of the CH$_2$NH$_2$—side chain of PBG combines with the carbon atom (marked *, figure 3) of a second PBG molecule, thus preserving the cyclic order. If acting alone, PBG deaminase catalyzes head to tail condensation, resulting in UPG I.

In head to head condensation, two PBG molecules combine through their CH$_3$NH$_2$ side chains, yielding a dipyrrole with the side groups inverted (A,P,P,A). When this condensation is effected chemically, ammonia and formaldehyde are produced. The side groups are in the inverted order A,P,P,A in ring D of UPG III, suggesting that head to head condensation has occurred. In the enzymatic production of UPG III, formaldehyde cannot be detected. Cosynthetase enters into a loose association with the deaminase and changes its mode of action so that the mixed enzyme reaction results in head to head condensation, a dipyrrole is formed and an active methyl group produced. Further condensation of a PBG takes place at this active methyl group giving a tripyrrole and active methyl group; finally, a fourth PBG is added and a closed ring tetrapyrrole formed. The products of

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<td>Uroporphyrinogen I</td>
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<td>Uroporphyrinogen III</td>
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<td>Porphyrin S - 411</td>
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A = CH$_2$COOH V = CH=CH$_2$
P = CH$_2$CH$_2$COOH Acr = CH=CH=COOH
M = CH$_3$ H = Unsubstituted hydrogen
the intermediate reaction do not escape from the enzyme (zipper effect). The cosynthetase is always in great excess of the deaminase, thus assuring continuous formation of UPG III. The deaminase-cosynthetase enzyme system is thus functionally a methyltransferase.

Stage Four

The fourth stage of the biosynthesis also occurs in the cytosol. UPG III is partially decarboxylated enzymatically to yield coproporphyrinogen III (CPG)\(^\text{30}\) (table I). Uroporphyrinogen decarboxylase converts the four acetic (a) side chains to methyl (M) groups sequentially, giving rise successively to porphyrins containing seven, six, five and finally four acid groups. In health, only trace amounts of the seven, six and five forms are found but considerable quantities may occur in some of the porphyrias, particularly in porphyria cutanea tarda, in which there is a deficiency of UPG decarboxylase. Of the 24 theoretical routes of decarboxylation between UPG III and CPG III, the one preferred biologically is clockwise, starting in ring D and progressing through rings A, B and C. The mechanism of the enzymatic decarboxylation is unknown.

Stage Five

In the fifth stage, coproporphyrinogen III is converted enzymatically to protoporphyrin IX (PP IX)\(^\text{31}\) (table I). The reactions take place in the mitochondrion. Two enzymes act sequentially, the first in two steps. Coproporphyrinogen oxidase catalyzes the oxidative decarboxylation of CPG III to protoporphyrinogen IX (PBG IX); protoporphyrinogen dehydrogenase completes the oxidation of protoporphyrin IX (PP IX).\(^\text{29}\) Oxidative decarboxylation converts the propionic acids (P) of rings A and B to vinyl (V). The P and V conversions take place in sequence, the first occurring in ring A to yield a tripropionic acid porphyrin called harderoporphyrinogen. This porphyrin is found normally in small amounts in bile, bone marrow, and erythrocytes (as harderoporphyrin). Its isomer (V in ring B) is not found in man. Two other recently discovered porphyrins are degradation products of harderoporphyrin, which do not occur in the biosynthetic pathway of heme. They are pemtotoporphyrin, in which V in ring A of harderoporphyrin is replaced by H, and porphyrin S 411 found in meconium, in which the V of ring A is replaced by an acrylic group (–CH=CHCOOH). In the decarboxylation process, CPG III is attached to the enzyme through the P side chain of ring A. Conversion of P to V then occurs. The harderoporphyrin formed remains attached to the enzyme but rapidly rotates anticlockwise to bring the P side chain of ring B into the active center of the enzyme. The second oxidative decarboxylation now takes place and the product (PPG IX) no longer having P groups is released from the enzyme. CPG oxidase has strict substrate specificity, the groups M, M, P, M being required in that cyclic order on the pyrroles, although the first M can be replaced by V, H, or ethyl (E).

Protoporphyrinogen dehydrogenase\(^\text{29}\) is a recently discovered enzyme which oxidizes PPG IX to PP IX. It will also oxidize, but much less efficiently, harderoporphyrinogen and PPG XIII, but PPG IX is the preferred substrate.

Stage Six

The final stage of heme biosynthesis is the insertion of Fe\(^{2+}\) into protoporphyrin IX to form heme\(^\text{37,61}\) (figure 2). The enzyme involved is ferrochelatase, and the reaction takes place in the mitochondrion. In vitro experiments on hepatic ferrochelatase have shown that its activity is inhibited by divalent cations such as Zn\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\) in amounts about equal to that of the PP. Full enzyme activity is restored by Cu\(^{2+}\) in much lower concentrations. Ferrochelatase activity requires reducing conditions provided in vitro by
thiol reducing agents and ascorbate and in vitro probably by reduced glutathione (GSH). The enzyme probably has similar requirements in the erythrocyte precursors. Ferrochelatase activity is subject to substrate inhibition by PP and to feedback inhibition by heme. Although ALA-S is the rate limiting enzyme of heme production, ferrochelatase plays a regulatory role.52

Specific inhibition of ferrochelatase by DDC (3,5-dicarboxyethyl-1,4-dihydrocollidine) and metals such as lead results in induction of ALA-S and accumulation of some porphyrin intermediates. It is probable that copper is required in vivo for the full enzymatic activity of ferrochelatase. It is believed that pyridoxal phosphate is a cofactor of ferrochelatase. Some of the effects of pyridoxine deficiency in man involving iron, e.g., ringed sideroblasts and accumulation of PP in these cells and in siderocytes, can be explained if pyridoxal phosphate is involved in ferrochelatase activity and is bound to the enzyme. The cofactor can then chelate ferrous iron and maintain it in the reduced state. Lead might compete with iron in this process, or might interfere with the binding of the coenzyme by the enzyme.

The enzymes, substrates and intermediates involved in heme biosynthesis must not be considered in isolation. Heme synthesis occurs in cells undergoing a wide range of other metabolic activities. In particular, heme is incorporated into heme proteins (hemoglobin, myoglobin, cytochromes, catalase, etc.) and is destroyed by heme oxygenase to produce bile pigments. Some of the enzymes require sulphhydryl compounds; therefore, enzymes concerned with glutathione metabolism may be indirectly involved in heme biosynthesis (e.g., glutathione reductase, glutathione synthetase, glutathione peroxidase28,40). Hydrogen peroxide and "active" oxygen are produced during mitochondrial activity and must be removed by appropriate oxidases, peroxidases, and superoxidases. It is possible that some of the effects of toxic metals, such as lead on heme biosynthesis, might be due to their interaction with these other enzyme systems.

**Lead Toxicity and Heme Biosynthesis**

The hematological manifestations of chronic lead poisoning vary widely in their severity and do not correlate well with blood lead concentrations. Anemia, although common, is usually of mild severity and hemoglobin concentrations rarely fall below 9 g per dl. The red cells are usually normocytic and normochromic, but may be microcytic and hypochromic, especially in children. The mean corpuscular hemoglobin concentration (MCHC) is only moderately reduced. There is usually some polychromasia and a mild reticulocytosis (2 to 7 percent). Basophilic stippling is occasionally found; however, when it is present, it is not pathognomonic of lead poisoning. There may be some anisocytosis and poikilocytosis, and occasionally a few nucleated red cells are seen. The osmotic fragility of the red cells is decreased, but their mechanical fragility is increased, and their life-span decreased. The bone marrow shows an erythroid hyperplasia and stippling of many nucleated red cells, some of which are ring sideroblasts. The anemia of lead poisoning may thus be classified as a secondary sideroblastic anemia. The hematological changes of lead poisoning are more pronounced in subjects having iron deficiency.

The chemical findings in the urine in lead poisoning, as they relate to heme biosynthesis, are5,25,52: increased delta-aminolevulinic acid (8-ALA) excretion (greater than 20 mg per l); increased coproporphyrin III (CP) excretion (greater than 0.5 mg per l); normal or slightly increased porphobilinogen (BG) excretion (normal: less than 2 mg per l).
However, as δ-ALA and CP excretion may be within normal limits in subjects with definite lead poisoning, these tests are of limited value in the diagnosis of lead poisoning or in the assessment of its severity.

Lead has been shown to depress the activity of three of the enzymes of the heme biosynthetic pathway, namely δ-aminolevulinate dehydratase (ALA-D), coproporphyrinogen oxidase (CPG-O) and ferrochelatase. ALA-synthetase activity is increased. The other enzymes (UPG III synthetase activity has not been investigated) are unaffected by lead poisoning. The mitochondrial enzymes (ALA-S, CPG-O, ferrochelatase) are assayed in extracts of leucocytes, the cytosolic enzymes in red cell hemolysates. Suitable assay methods are available, but, with the exception of ALA-D, are too involved for use in the routine clinical laboratory. The sensitivity of ALA-D to extraneous lead contamination makes measurement of the activity of this enzyme unreliable, unless stringent precautions are adopted to exclude lead.

Inhibition of ferrochelatase explains the increased protoporphyrin IX (PP) seen in the red cells in lead poisoning; inhibition of ALA-D and increase in ALA-S account for the increased excretion of δ-ALA; inhibition of CPG-O explains the increase in CP found in red cells and urine. However, it is not convenient to assay heme enzymes routinely in the diagnosis of lead poisoning because of technical difficulties and because all of the enzymes would have to be measured to establish the pattern characteristic of lead poisoning. As a practical alternative, attention has been focused on the measurement of erythrocyte protoporphyrin (EPP) as an indicator of lead poisoning.

**Erythrocyte Protoporphyrin**

It is now well established that EPP is increased in lead poisoning subjects. The methods used for measuring EPP vary in detail, but essentially involve extraction of porphyrins into an ethyl acetate-acetic acid mixture and extraction of the porphyrins from the organic solvent with hydrochloric acid. According to the conditions used, varying amounts of protoporphyrin IX, coproporphyrin III, and uroporphyrin I are extracted and, possibly, other porphyrins. Protoporphyrin is measured fluorimetrically by suitable choice of excitation and emission wavelengths. The porphyrins were named free erythrocyte protoporphyrin (FEP) in the belief that they are metal-free (iron protoporphyrin does not fluoresce under these conditions). Piomelli's micromethod for FEP, which is widely used in its original form or with some modifications, measures PP and CP, although the latter is a relatively minor constituent. Coproporphyrin I is used as a standard and an empirical correction factor is employed to convert CP into PP fluorescence. Although the method is simple, it does not give reproducible answers in different hands, probably because of incomplete extraction of porphyrins, the presence of other porphyrins besides PP, and the variability of the correction factors with the fluorometer used. FEP methods must therefore be considered as screening methods. In a recent interlaboratory comparison of EPP using extraction procedures, the coefficient of variation obtained from 45 laboratories was 16.8 percent for a low value (0.72 mg per l) and 18.1 percent for a higher value (1.94 mg per l), although the means were close to those of the reference laboratories. The c.v.s. for the seven reference laboratories were, respectively, 9.4 percent and 9.3 percent. It is clear that considerable improvement in methodology is required if FEP is to be used as a diagnostic test.

As has been mentioned in the historical introduction, Lamola and coworkers showed that the erythrocyte protopor-
phyrin in lead poisoning was zinc protoporphyrin (ZPP)\textsuperscript{39} and devised a simple analytical method for its measurement.\textsuperscript{36} Because the zinc is readily removed in the usual extraction procedures, it is convenient to measure ZPP in red cell hemolysates or whole blood without prior extraction. Three Hematofluorometers are commercially available, employing the principles of front surface illumination reflectance spectrofluorometry.\textsuperscript{9,10} Excitation is at 420 to 430 nm and emission is measured at 580 to 680 nm. The standard is a stable dye (Rhodamine B dissolved in Krylon Black Enamel) deposited on a slide. Incident light illuminates in turn, for a fixed period, a blank slide (background), the standard, and the sample. The intensities of the emitted light ($I_o$, $I_s$, and $I_b$, respectively) are measured and $R = R_s (I_b - I_o) / (I_s - I_o)$ calculated. $R_s$ is the calibration constant of the instrument, determined by the manufacturer using bloods of known ZPP concentrations. The intensity of the light emitted from the sample depends on the amount of light it absorbs and the amount emitted by the fluorescent porphyrins. It is in reality proportional to the ratio of ZPP to hemoglobin in the sample. However, to conform to the method used in reporting results of extraction methods, the instrument displays the ZPP concentration in $\mu g$ per dl of blood, assuming a hematocrit of 35 percent (for children) and presumably a normal hemoglobin concentration. In the interlaboratory comparison referred to, the c.v.s were 11.9 percent and 9.9 percent at the low and high concentrations of ZPP for 34 laboratories using Hematofluorometers, but the means were about 10 percent lower than the reference values.

Errors arising from different hematocrit and hemoglobin concentration can be corrected, but others may occur. For example, the blood must be fully oxygenated (reduced hemoglobin and methemoglobin absorb different amounts of light from oxyhemoglobin); otherwise, a marked reduction in apparent ZPP concentration is reported. Many substances may interfere with fluorescence measurements. In particular, bilirubin has been reported to give erroneously high values of ZPP.\textsuperscript{13} Using the best combination of filters, each mg per dl of bilirubin increases the apparent ZPP concentration by 6 $\mu g$ per dl,\textsuperscript{37} but the error may be larger with some instruments. No doubt other sources of error will be reported as the instruments become more widely used. Nevertheless, they are of great value as field instruments in the detection of possible lead poisoning.

Interpretation of FEP and ZPP Concentrations\textsuperscript{*}

FEP is, to a small extent, age and sex dependent, being higher in young children and females.\textsuperscript{33} Most investigators agree on a range in adults of 35 to 65 $\mu g$ per dl of red cells, with 80 $\mu g$ per dl as the three standard deviation upper limit and values greater than 150 $\mu g$ per dl indicative of lead poisoning (assuming a normal hemoglobin concentration\textsuperscript{18,57}). In 20 normal infants (hemoglobins between 11.0 and 13.1 g per dl) FEP ranged between 11.6 and 35.4 $\mu g$ per dl of whole blood; mean, 23.1; s.d., 5.4 $\mu g$ per dl, corresponding to an upper limit of about 100 $\mu g$ per dl of red blood cells.\textsuperscript{46} A better index is the ratio of FEP (or ZPP) to hemoglobin. This ratio had a mean value of 1.9 $\mu g$ FEP per g of Hb in the same 20 infants. Ratios greater than 17.5 $\mu g$ per g are found in severe lead poisoning and values over 5.5 $\mu g$ per g are suggestive of it.\textsuperscript{46}

FEP and ZPP and the FEP/Hb\textsuperscript{46} or FEP/Heme\textsuperscript{35} ratio are increased in many other conditions besides lead poisoning.

* FEP refers to the erythrocyte protoporphyrin measured using conventional extraction procedures. ZPP refers to the erythrocyte protoporphyrin measured (as described above) directly in red cells or whole blood, or after the special extraction techniques.\textsuperscript{48}
especially iron deficiency anemia.\textsuperscript{46,57} FEP is raised in the rare hereditary disorder, erythropoietic protoporphyrina,\textsuperscript{21} and also in febrile chronic infections in which values as high as 400 \(\mu g\) per dl of red cells may be found.\textsuperscript{32} Values may be raised in the post hemorrhagic state. In sheep, high values of FEP have been reported in vitamin \(B_{12}\) and copper deficient animals\textsuperscript{8} and in rabbits with vitamin E deficiency.\textsuperscript{17}

Recently, elevated values have been found in patients suffering from Friedreich's ataxia and some other ataxias.\textsuperscript{41}

In essence, erythrocyte protoporphyrin increases in conditions in which there is increased erythropoiesis\textsuperscript{49} and as a result of inhibition of certain enzymes of the heme synthetic pathway, either because of an inborn error of metabolism or because of toxic substances. In the case of lead poisoning, there is a reciprocal relationship between blood lead concentration and FEP and ZPP concentration. However, because of the large experimental errors involved in the measurement of FEP and ZPP, and also of blood lead, borderline elevations must be interpreted with caution. The importance of erythrocyte protoporphyrin is that its concentration reflects the continued action of lead on the hematopoietic system; blood lead is more related to the intake of lead and its transport to various locations. FEP and ZPP are therefore more likely to be related to the severity of lead poisoning than is blood lead. Experience has shown that erythrocyte protoporphyrin measurement is a more sensitive indicator of lead poisoning than is the measurement of urinary porphyrins or delta-aminolevulinic acid and is easier to carry out than the assay of ALA-D activity.

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