Zinc Protoporphyrin in the Erythrocytes of Patients with Lead Intoxication and Iron Deficiency Anemia

Abstract. *The fluorescent porphyrin in the erythrocytes of patients with lead intoxication or with iron deficiency anemia is zinc protoporphyrin that is bound to globin moieties, probably at heme binding sites.*

It is well known that large amounts of protoporphyrin-IX (PP) can be extracted from the erythrocytes of patients with lead intoxication (PbI) or severe iron deficiency anemia (FeD) with the use of acidic solvents that extract very little PP from the erythrocytes of normal controls (*1–10*). The literature concerning these disorders refer to this extractable PP as "free erythrocyte protoporphyrin" (*1–11*). The term "free erythrocyte protoporphyrin" implies that the PP is present as the free base (unchelated) in the erythrocytes. This is supported by the fact that the majority of erythrocytes from patients with severe and chronic PbI or FeD exhibit red fluorescence when excited with blue light (*1, 11*). Mature erythrocytes from normal controls do not show this fluorescence (PP bound to iron is not fluorescent).

We report here that the fluorescent PP in erythrocytes of patients with PbI or FeD is, in fact, not "free" but is chelated with zinc.

As part of an investigation (*12, 13*) of the molecular basis for the difference in cutaneous photosensitivity among the various disorders in which concentrations of "free erythrocyte protoporphyrin" are elevated, we examined the fluorescences of diluted whole blood, suspensions of washed erythrocytes, and hemoglobin purified by gel filtration (Sephadex G100) from subjects with PbI, FeD, and erythropoietic protoporphyria (EPP). Representative spectra are shown in Fig. 1. The diluted blood, erythrocytes, and hemoglobin from patients with either PbI or FeD exhibited identical porphyrin-like spectra with the position of the 0′-0 (zero-zero) band maximum at 594 nm. In contrast, those from patients with EPP exhibited fluorescence spectra with 0′-0 band maxima at 625 nm. (Blood from EPP patients usually shows a small amount of 594-nm fluorescence.) The excitation spectrum for the EPP-type fluorescence (Soret maximum, 397 nm) was also different from that for the PbI type (Soret maximum, 424 nm). In contrast to these findings the fluorescence spectra of extracts made with acidic solvents (typically a mixture of ethyl acetate, acetic acid, and 1.5*N* HCl) were identical whether the extracted blood came from a patient with EPP, PbI, or FeD. The spectrum [0′-0 band maximum, 606 nm; excitation maximum (Soret), 405 nm in 1.5*N* HCl] is identical to that of PP in that solvent.

The above observations indicate that most of the fluorescent PP in blood from patients with EPP is bound in a different way from that in blood from patients with PbI (*12, 13*). (Unless otherwise indicated, the observations concerning the fluorescent porphyrin associated with blood from PbI patients are true for blood from FeD patients as well.) In both PbI and EPP, 98 to 100 percent of the whole blood fluorescence (or extractable PP) is found in the erythrocyte fraction, and more than 95 percent elutes identically with the hemoglobin band upon gel filtration chromatography (Sephadex G100). These and other data suggested that in blood from patients with either EPP or PbI most of the fluorescent PP is bound to hemoglobin (or some tetrameric globin) and require that the manner in which this porphyrin is bound is different in the two cases (*12, 13*).

We were able to show that PP in its free base form bound to hemoglobin at an extra heme site (or sites) exhibits the type of fluorescence observed in EPP (emission maximum, 625 nm; excitation maximum, 397 nm). As demonstrated by other workers (*14*), we also found that PP bound to a globin chain at the heme site fluoresces in a similar way (emission, 625 nm; excitation, 403 nm). On the other hand, we were not able to obtain a compound that emits at 594 nm by combining PP, globin, and heme in any way. On the basis of chromatographic behavior we were able to discount the possibility that the 594-nm emission arises from PP combined with fetal globin or other altered globins that are sometimes associated with anemias (*12*). Finally, it appeared that no ionized form of PP could account for the emission at 594 nm. We concluded that the fluorescent PP of the PbI type is probably not "free" but chelates a metal ion.

The only strongly fluorescent metalloporphyrins contain metal ions that are diamagnetic and are not too heavy (*15, 16*). By these criteria the possibilities immediately narrow to zinc and magnesium, both of which are plentiful in the erythrocyte (*17*). We pursued this idea because of the recent paper of Leonard *et al.* (*14*), who reported that the fluorescence spectrum of ZnPP-globin has a 0′-0 band maximum at 593 nm.

Zinc protoporphyrin (ZnPP) and magnesium protoporphyrin (MgPP) were each added to apohemoglobin by mixing the porphyrin and protein (in excess) in 0.01*M* phosphate buffer (*p*H 7.4) and allowing the solution to stand overnight at 4°C. (The porphy-

Table 1. Fluorescence parameters of protoporphyrin, zinc protoporphyrin (ZnPP), and magnesium protoporphyrin (MgPP) in various environments. Abbreviation: Hb, hemoglobin.

Protoporphyrin states	Wavelengths (nm) of maxima*	
	Emission	Excitation
Erythrocytes or Hb from PbI or FeD patients†	594	424
ZnPP-globin‡	594	423
MgPP-globin	594	420
PP-globin	625	403
Ethanol extract of Hb in PbI	589	415
ZnPP in ethanol	589	415
MgPP in ethanol	594	413
PP in ethanol	632	403
Ethanol extract of Hb in PbI, dried, taken up in buffer (0.01*M* phosphate, *p*H 7.4)	585	407
ZnPP in buffer	584	406
MgPP in buffer	596	411
PP in buffer	619	400
Ethanol extract of Hb in PbI, dried, taken up in pyridine	594	424
ZnPP in pyridine	593	424
MgPP in pyridine	604	427
PP in pyridine	635	408

* The 0′-0 band in the fluorescence spectrum and the Soret band in the fluorescence excitation spectrum. Precision ± 1 nm; wavelengths not corrected for spectral response of the spectrofluorimeter, a Perkin-Elmer-Hitachi MPF2 equipped with a Hamamatsu 446UR photomultiplier tube. † Diluted 1 : 400 from normal hematocrit. ‡ Solutions of porphyrins $\leq 10^{-6}M$.

rins bind to the globins at the heme binding sites.) The solutions were diluted and their fluorescence spectra were recorded (Table 1). As found by Leonard *et al.* (*14*), ZnPP-globin emits with a maximum at 594 nm, excitation maximum, 423 nm—in good agreement with the data for the hemoglobin fraction of blood from PbI patients. The MgPP-globin showed very similar spectral characteristics—emission maximum, 594 nm; excitation maximum, 420 nm. The small but real difference in the positions of the excitation maxima suggested that the fluorescent porphyrin in blood from PbI patients is ZnPP and not MgPP.

We were able to distinguish between ZnPP and MgPP by their fluorescence spectra in various solvents; therefore we attempted the extraction of the purported metalloporphyrin from PbI hemoglobin. Ethanol was a convenient and efficient solvent for this purpose.

Packed washed erythrocytes from PbI patients, which had at least 60 μg of extractable PP per 100 ml of blood or very concentrated solutions of the hemoglobin fraction from such cells, were mixed with ten volumes of absolute ethanol, shaken vigorously for 1 minute, and centrifuged. The clear, very lightly colored supernatant fraction contained 50 to 80 percent of the fluorescent porphyrin. The spectrum of the fluorescence exhibited by the ethanol extracts of erythrocytes from PbI patients was identical to that of an ethanol solution of ZnPP and different from that of an ethanol solution of MgPP (Table 1). Ethanol extracts of hemoglobin from PbI patients were evaporated to dryness with a stream of dry nitrogen, and the residue was taken up in 0.01*M* phosphate buffer (*p*H 7.4). The latter solutions exhibited fluorescence with a spectrum identical to that of a solution of ZnPP in buffer, but different from that of MgPP in buffer (Table 1). Ethanol extracts of whole erythrocytes contain a large amount of lipid. When these extracts are dried and taken up in buffer, the fluorescence resembles that of ZnPP in detergent micelles. Finally, dried ethanol extracts of hemoglobin, from PbI patients, taken up in pyridine fluoresce with a spectrum identical to that of ZnPP in pyridine but different from that of MgPP in pyridine (Table 1).

In order to support the spectroscopic evidence identifying ZnPP as the fluorescent porphyrin in blood from PbI patients, we performed trace metal analyses by atomic absorption spectrophotometry. The concentration of fluorescent porphyrin in the erythrocytes in severe cases of PbI is typically on the order of 0.1 percent of the heme concentration and small compared to the concentrations of many metal ions, including magnesium, zinc, and copper. Therefore, to facilitate removal of extraneous metal ions we performed the metal analyses on ethanol extracts of hemoglobin from PbI patients. The following procedure worked well. Erythrocytes from these patients were washed with buffered saline and then lysed in ten volumes of distilled water. After the stromata were removed by centrifugation the lysate was concentrated (vacuum dialysis) to about the original volume of cells. This preparation was extracted with ten volumes of absolute ethanol as described above. The lightly colored extract was stirred for 10 minutes with one-sixth its volume of Chelex-100 (Bio-Rad; 50–100 mesh, sodium form) to remove unbound metal ions. The ethanol solution was decanted and analyzed. Throughout the procedure fluorescence spectra were recorded to check on the mass balance of the fluorescent porphyrin and to ensure that the spectrum remained characteristic of the original whole blood. Fluorimetry (in 1.5*M* HCl) was used to determine the concentration of porphyrin in the final ethanol solution. In this way we demonstrated that zinc was present in an amount close to stoichiometry with the porphyrin in the extract; much less magnesium was present and only insignificant amounts of lead, manganese, nickel, cobalt, cadmium, and copper. For example, when 6.5 ml of erythrocytes, from PbI patients, containing 700 μg of "free erythrocyte protoporphyrin" per 100 ml of erythrocytes was extracted, we found 4.1 ± 0.2 μg (0.063 μm) of zinc in the extract compared with 3.6 ± 0.2 μg of zinc expected on the basis of fluorimetry; we also found 0.13 μg (0.0054 μm) of magnesium and much smaller amounts of the other metals for which we analyzed.

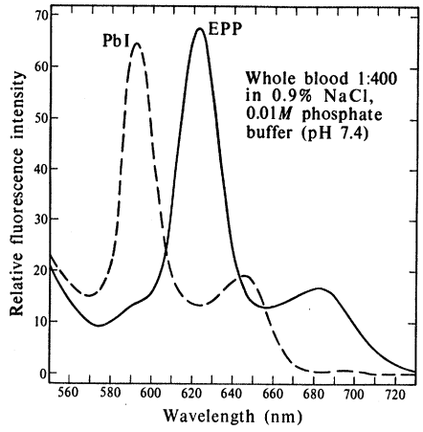

Fig. 1. Fluorescence spectra of whole blood diluted 1 : 400 with buffered saline. The dashed curve is the spectrum obtained from the blood of patients with lead intoxication (PbI); blood of patients with iron deficiency anemia fluoresces similarly. The solid curve is the spectrum obtained from the blood of patients with erythropoietic protoporphyria (EPP).

We conclude that the porphyrin species, which emits at 594 nm, found in blood of patients with PbI or FeD is predominantly ZnPP. This ZnPP elutes with the hemoglobin upon gel filtration chromatography and elutes with the α- and β-chain fractions if the hemoglobin is treated to separate the chains (*12*). Thus it appears that the ZnPP is bound to globin moieties, probably at heme binding sites.

We explain the occurrence of ZnPP in cases of PbI and FeD in the following ways. Heme synthesis is inhibited in cases of FeD anemia because of the iron deficiency and in PbI because of inhibition of ferrochelatase activity by lead (*7, 18, 19*). If PP and globin syntheses proceed normally, then metal-free PP and "vacant" heme sites on globins accumulate. It is known that most ferrochelatase systems can catalyze the insertion of Zn^{2+} into PP (*20–22*). This could explain the ZnPP in FeD. In PbI one possibility is that zinc chelatase activity is not inhibited proportionately with ferrochelatase activity. Mammalian chelatases do not appear to use Mg^{2+} (*23*). Nonenzymic production of ZnPP is another way to explain the observations: With a reduced rate of enzymic iron insertion into PP the nonenzymic chelation of zinc might compete. The Zn^{2+} is incorporated into PP in an aqueous environment at an appreciable rate, whereas Mg^{2+} is not (*24*).

The ratio of PP to "vacant" heme binding sites may explain the difference between PbI and FeD, and EPP. Zinc protoporphyrin may be stabilized by binding at heme sites. In EPP, PP synthesis is elevated but heme synthesis is normal (*24*). Consequently, there are relatively few "vacant" heme sites and so only a small amount of the accumulated PP is bound as ZnPP. The remainder of the PP is bound as the free base at other sites which do not favor ZnPP. An additional possibility is that ZnPP is an intermediate in heme bio-

synthesis which accumulates in PbI and FeD. The defect in EPP is different and allows the earlier intermediate PP to accumulate.

Finally, we wish to point out a clinical application of our observations. We and Joselow (*25*) have shown that measurement of the fluorescence, at 594 nm, of blood directly (without any extraction steps) can serve as the simplest and most specific screening test for PbI.

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References and Notes

1. A. A. H. Van Den Bergh and W. Grotepass, *Klin. Wochenschr.* **12**, 586 (1933).
2. R. J. Watson, E. Decker, H. C. Lichtman, *Pediatrics* **21**, 40 (1958).
3. H. C. Lichtman and F. Feldman, *J. Clin. Invest.* **42**, 830 (1963).
4. C. J. Watson, *A.M.A. Arch. Intern. Med.* **99**, 325 (1957).
5. S. Granick, S. Sassa, J. L. Granick, R. D. Levere, A. Kappas, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2381 (1972).
6. L. P. Kammholz, L. G. Thatcher, F. M. Blodgett, T. A. Good, *Pediatrics* **50**, 625 (1972).
7. J. J. Chisolm, Jr., *J. Pediatr.* **64**, 174 (1964).
8. S. Piomelli, B. Davidow, V. F. Guinee, P. Young, O. Gay, *Pediatrics* **51**, 254 (1973).
9. S. Schwartz, M. H. Berg, I. Bossenmaier, H. Dinsmore, in *Methods of Biochemical Analysis*, D. Glick, Ed. (Interscience, New York, 1960), vol. 8, pp. 221–294.
10. L. Wranne, *Acta Pediatr. Scand.* **49**, 1 (1960).
11. J. A. Whitaker and T. J. Vietti, *Pediatrics* **24**, 734 (1959).
12. A. A. Lamola, T. Yamane, S. Piomelli, M. Poh-Fitzpatrick, unpublished data.
13. S. Piomelli, A. A. Lamola, M. Poh-Fitzpatrick, E. Carlos, C. Seaman, L. Harber, unpublished data.
14. J. J. Leonard, T. Yonetani, J. B. Callis, *Biochemistry* **13**, 1460 (1974).
15. J. E. Falk, *Porphyrins and Metalloporphyrins* (Elsevier, New York, 1964), pp. 85–87.
16. R. S. Becker, *Theory and Interpretation of Fluorescence and Phosphorescence* (Wiley-Interscience, New York, 1969), pp. 190–197.
17. E. J. Underwood, *Trace Elements in Human and Animal Nutrition* (Academic Press, New York, ed. 2, 1962), p. 157.
18. M. Grinstein, R. M. Bannerman, C. V. Moore, *Blood* **14**, 476 (1959).
19. H. C. Schwartz, G. E. Cartwright, E. L. Smith, M. M. Wintrobe, *ibid.*, p. 486.
20. See G. H. Tait, in *Porphyrins and Related Compounds*, T. W. Goodwin, Ed. (Academic Press, New York, 1968), pp. 31–33.
21. See G. S. Marks, *Heme and Chlorophyll* (Van Nostrand, London, 1969), pp. 146–147.
22. H. Oyama, K. Sugita, Y. Yoneyama, H. Yoshikaya, *Biochim. Biophys. Acta* **47**, 413 (1961).
23. R. F. Labbe and N. Nubbard, *ibid.* **52**, 130 (1961).
24. J. N. Phillips, in *Comprehensive Biochemistry*, M. Florkin and E. H. Stotz, Eds. (Elsevier, Amsterdam, 1963), vol. 9, p. 34.
25. A. A. Lamola, M. Joselow, T. Yamane, *Clin. Chem.*, in press.
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Arginine Vasotocin: Effects on Development of Reproductive Organs

Abstract. *Immature 25-day-old mice were injected daily with 1 microgram of arginine vasotocin for 3 or 4 days and killed 24 hours after the last injection. The ovaries were 30 percent smaller in treated females than in controls. The ventral prostates and accessory organs (seminal vesicles and coagulating glands) were less than half the size of these structures in control males. Similar results were observed when 15-day-old mice were given similar injections and killed 2 weeks after the last injection; furthermore, testis weights were 28 percent smaller than those of controls. It is speculated that arginine vasotocin, which has been found in mammalian pineal glands, might mediate effects of the pineal gland on normal sexual development.*

Arginine vasotocin (AVT), an octapeptide, has been identified in the mammalian pineal gland and proposed to be the antigonadotrophic product of that gland (*1*). Pavel *et al.* (*2*) reported that this cyclic peptide is a million times more potent than melatonin, the putative pineal hormone, in inhibiting the compensatory ovarian hypertrophy that occurs in adult female mice after unilateral ovariectomy. In this report we present experimental evidence that treatment of normal immature mice with AVT results in smaller reproductive organs in both males and females.

Immature Swiss-Webster mice were obtained from Hilltop Lab Animals, Inc. (Scottdale, Pennsylvania) or bred from stock derived from that source. In these animals, vaginal patency occurs at 30 to 35 days of age and reproductive competence for both males and females is attained by 60 days of age. The animals were maintained in an environment with automatically controlled lighting that provided 14 hours of light per day. In the first experiment, groups of 25-day-old female and male mice received daily intraperitoneal injections for 3 and 4 days, respectively, either of 1 μg of AVT in 0.1 ml distilled water or of diluent alone (*3*). Their reproductive organs were examined 24 hours after the last injection. In addition, two groups of normal male and female mice were necropsied at 25 days of age. Compared to diluent treatment of age-matched mice, AVT treatment decreased the weight of the ovaries ($P < .001$) in female mice and accessory organs (seminal vesicles and coagulating glands) ($P < .01$) and ventral prostates ($P < .001$) in male mice (Fig. 1). In the period between days 25 and 28, ovaries of control mice increased in weight by 22 percent whereas AVT treatment during this period prevented this normal developmental growth (*4*).

In the second experiment, five litters of mice were reduced to eight pups per mother on the day of birth and random-

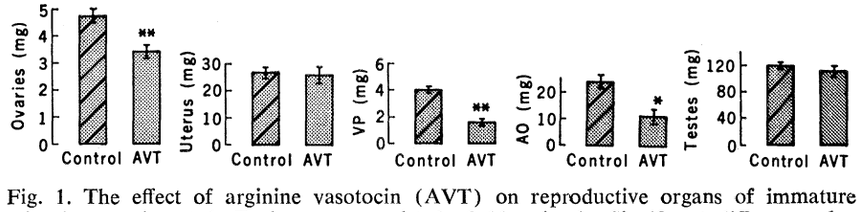

Fig. 1. The effect of arginine vasotocin (AVT) on reproductive organs of immature mice in experiment 1. Each group consisted of 11 animals. Significant differences between AVT groups (gray bars) and controls (striped bars) are indicated as follows: * $P < .01$; ** $P < .001$. *VP*, ventral prostate; *AO*, accessory organs.

Table 1. Weights of reproductive organs of immature mice treated with AVT in experiment 2. Data for each group are means ± standard error for eight to ten mice.

Female group	*Body weight (g)*	*Ovaries (mg)*	*Uterus (mg)*	
Control	20.3 ± 0.3	4.93 ± 0.20	17.9 ± 2.1	
AVT	18.5 ± 0.6	3.44 ± 0.23*	15.2 ± 0.8	
Male group	*Body weight (g)*	*Testes (mg)*	*Ventral prostate (mg)*	*Accessory organs (mg)*
Control	24.3 ± 0.5	166.5 ± 3.5	4.15 ± 0.09	22.4 ± 1.1
AVT	23.3 ± 0.5	120.5 ± 4.1†	2.34 ± 0.19†	15.0 ± 1.0*

* $P < .01$. † $P < .001$.