To the Graduate Council:

I am submitting herewith a dissertation written by Thomas L. Walden, Jr., entitled "The Elevation of Blood Levels of Zinc Protoporphyrin in Mice Following Whole Body Irradiation." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Radiation Biology.

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THE ELEVATION OF BLOOD LEVELS OF ZINC PROTOPORPHYRIN IN MICE FOLLOWING WHOLE BODY IRRADIATION

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Thomas L. Walden, Jr.

June 1983

Dedicated to my parents,

Tom and Betty

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This manuscript commemorates the completion of 23 years of formal education, 79.31034% of my 29 years. Twenty-three years of draining the public school system, the State of North Carolina, the State of Tennessee, the Cystic Fibrosis Foundation, my parents, the Bank of Maryville, and me. I never thought it would take me this long. After 23 years, I now stand at the boundary line, ready to enter the "real world," ready to be a productive, if not "burnt out," member of society. Thank God this is it. And in addition to God, there are several other people that I would also like to thank.

A graduate student entering a new lab is much like a stray dog or an orphaned child being taken off the streets, sometimes they get good homes and sometimes they are neglected and abused. I was lucky enough to receive a good home. My respect and admiration for Dr. Farkas is second only to that for my father. I have acquired valuable skills and knowledge under his direction. My acquisition of these skills did not come cheaply to either of us--for every successful experiment there were probably 10 that were wasted, botched, or unproductive. He has never ridiculed my ideas, instead provided me with enough room to allow me to feel independent but with the proper amount of guidance to keep me moving forward and always ready to lend me a hand when I got in over my head. I can never repay the debts of gratitude or finance which I owe him. I hope that one day I may be privileged to return his kindness by training

graduate students in my lab in the skills and in the manner that Dr. Farkas has taught me.

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ABSTRACT

Elevation of zinc protoporphyrin (ZPP) levels in the blood has served as an indicator of lead poisoning and iron deficiency anemia for many years. We have discovered that sublethal doses of whole-body irradiation with X-rays also elevates ZPP two- to three-fold over normal levels. The ZPP level does not begin to increase until days 12 to 14 post-irradiation and peaks between days 18 to 20 before returning to normal levels between days 28 to 35. Increasing the radiation dose delays the onset of the rise in ZPP but does not affect the magnitude of the elevation. At lethal doses, ZPP elevation is not observed. Neither of the two previously described mechanisms which cause elevations of ZPP, namely iron deficiency and inhibition of ferrochelatase, are responsible for the radiation induced elevation of ZPP. The elevation of ZPP appears to be correlated with the recovery of the hematopoietic system from radiation injury.

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INTRODUCTION

The final reaction in the biosynthesis of heme is the combination of Fe^{2+} with protoporphyrin IX catalyzed by ferrochelatase. Zinc can also combine with protoporphyrin IX, either spontaneously or enzymatically, to form zinc protoporphyrin (ZPP) (1,2). The enzymatic reaction between protoporphyrin IX and Zn^{2+} is competitively inhibited by Fe^{2+} (1). The non-enzymatic reaction with Zn^{2+} is actually slightly faster than with Fe^{2+} (2). ZPP is synthesized at a much slower rate than heme and like heme can be bound to globin (3) where it remains throughout the lifetime of the red-blood cell (4). Unlike heme, ZPP does not participate in oxygen transport. To date, only two pathological conditions are known to cause elevation of ZPP: lead poisoning and iron deficiency anemia (5). Lead inhibits ferrochelatase and, during lead poisoning, ZPP is formed presumably by a nonenzymatic reaction (5). ZPP has a unique fluorescence spectrum, and a facile rapid assay has been developed to measure ZPP from a drop of whole blood in seconds (6-8). A fluorimeter designed exclusively for the determination of ZPP is used by public health agencies for screening children in urban areas who are at risk for lead poisoning (9,10) and for determining whether blood donors have an iron deficiency (11). The technique is also widely used in lead industries to screen for occupational plumbism (7,12-15). The use of ZPP measurements as a clinical and field diagnostic aid has increased over the last few years with some laboratories performing up to 400 assays per day (8).

We report here a third condition which causes ZPP to become elevated, namely whole-body X-irradiation. Our results show that the elevation of ZPP is related to the recovery of the hematopoietic system from radiation damage.

I. RECOVERY OF HEMATOPOIESIS FOLLOWING RADIATION DAMAGE

Introduction

The recovery of the bone marrow from radiation is important in radiotherapy (16,17), civil defense (18), and reaction planning for radiation accidents where it is one of the limiting factors since hematopoietic death occurs at lower doses than those observed for the other acute radiation syndromes. The present understanding of the processes and effects are based on the accumulation of 80 years of research (16,19-24). Quantitation of the effects reported in studies carried out prior to 1928 are hampered due to the lack of standardized dosimetry techniques available before that time (19). Radiation induced changes in the cellular composition of the peripheral blood are well documented (19-21,24) and, in general, at least 300 rads are required to affect the hematocrit in most species (25), although some small lymphocytes can be destroyed by as little as 25r (26-28). The effects on platelet numbers over the range 100-400r (16) and on the reticulocyte numbers over the range 12-900r (29) are proportional to the dose. Changes in the peripheral blood do not necessarily reflect the true recovery status of either the bone marrow or the spleen since the marrow may be less than 2/3 recovered even though the peripheral blood has returned to normal (16,30,31). In these cases, the bone marrow is compensated for by increased ectopic hematopoiesis in the spleen (32-34).

Irradiation of the bone marrow may have two effects: damage to the stem cells and/or damage to the microenvironment responsible for stem cell maintenance (16,17). Two basic populations of stem cells are present in the bone marrow, the hematopoietic and the stromal stem cells (35). Hematopoietic stem cells give rise to blood cells and platelets, while stromal stem cells differentiate into fibroblasts and other marrow cells responsible for supporting the proliferation and development of hematopoietic cells. Damage to the macroenvironment usually occurs in one of four basic ways: damage to the stromal cells, damage to the blood vasculature (usually a late irradiation effect) (30,36), altering the levels of humoral regulating factors (for example, erythropoietin) (7,20,22), and by alteration of cell surface components, in turn modifying intercellular communications which regulate hematopoiesis (16). The production of circulating factors possessing regulatory properties may explain the reports of radiation induced abscopal effects (31,33,38,39) such as the depression of marrow activity in the shielded femur of an otherwise whole-body irradiated animal (33). While large doses of radiation have been found to impair hematopoiesis, small doses, unlike the hormetic effect of some poisons, do not stimulate hematopoiesis (38).

Radiation Damage to and Recovery of the Bone Marrow

Radiation may affect red bone marrow by either cellular destruction and/or alteration of the microenvironment (16,17). The $LD_{50/30}$ for bone marrow death is 500 rads (16), although some species such as guinea pigs ($LD_{50/30}$ = 175 rads) (40) are more sensitive.

Generally, with doses less than 50r, the only visible effect on the bone marrow is the death of some small lymphocytes (26-28, 41), although several cell population and biomedical changes are detectable by special techniques. In humans, 150 rads given in doses of 15 rads per day depresses red blood cell counts although a single acute dose of 150 rads does not (42). Exposure of rats to a smaller fraction for longer periods of time, 1r/day for 1-2 years, did not produce observable hematological changes (21).

Immediately after radiation exposure, an increasing number of cells in the marrow exhibit pyknosis and fragmented nuclei (19) with the greatest damage appearing 2-5 hours post irradiation (21). The largest amount of damage, as scored by chromosomal aberrations in rats receiving 50 rads/day, occurred on the first day (43). Cessation of mitosis usually occurs within 30-45 minutes after doses greater than 200 rads (16,28,41). Within the first 2 hours after irradiation, there is an increased stimulation of hemoglobin synthesis which returns to normal rates within 24 hours (22). Ferrochelatase activity is not affected, although doses as low as 50r to rats (22) or 5r to guinea pigs (44) can inhibit [^{59}Fe] uptake by circulating red-blood cells. Most erythrocyte and hemoglobin changes require doses above 200r to be observed (21). There is a decrease in cellularity in the bone marrow (45) following irradiation which results from cellular destruction and from maturation depletion of differentiating cells. In rabbits receiving 800r, erythropoiesis is completely absent in the marrow within 72 hours (20).

Removal of the cellular debris by phagocytes already present in the marrow begins immediately after irradiation and is aided by an infiltration of additional phagocytic cells during the first couple of hours. Depending on the degree of damage, debris removal may be accomplished in several hours to a day (21). The extravascular blood which fills the resulting empty spaces (46) is slowly displaced by lipocytes which begin to accumulate on the first day, reaching peak numbers by the seventh day post irradiation (46-49). These lipocytes contain more triglycerides and less arachidonic acid than similar cells present in the marrow before irradiation (22,47,48,50). The overall pattern for regeneration, whether by local or migratory cells, involves first an accumulation of lipocytes into fat spaces in the sinusoidal cavities and an increase in reticular cell activity (36). In some cases, the lipocytes replace a gelatinous intercellular substance which may form during the post irradiation period (41). The accumulation of lipocytes is followed by the growth of trabecular bone into the marrow cavity (23,49). The trabecular bone is then reabsorbed and small colonies of hematopoietic and stromal stem cells reappear around the areas of reabsorption and slowly branch out to restore marrow activity (23,49,51,52). Reabsorption of the trabecular bone is a slow process, and traces have been observed up to 50 days after regeneration is initiated (52). During the first or second week, there is usually an abortive recovery of hematopoiesis (24). In rabbits given up to 2000r, the abortive recovery occurs between the second to the eleventh days post irradiation (20,24). Myeloid cell proliferation

recovers first because the microenvironment in the bone marrow preferentially favors myeloid cell formation over erythropoiesis (37). When erythropoiesis resumes, the early erythroblasts have constricted nuclei (41) and there is an increase in the number of stippled cells (53). The average red blood cell size also increases because of the increased number of reticulocytes in circulation. With local doses below 1000r, the marrow can make substantial gains toward normal levels by the fourteenth day post irradiation, after which it plateaus and may require several months to return to normal (31, 54). This plateau may be due to splenic compensation at about this time removing some of the stimulus from the bone marrow to recover (54). Six months after receiving 5000r local irradiation, radioiron uptake in mice was 15% of normal and cellularity 30%, while in mice receiving 1000r, these values were 67% and 76%, respectively (33). In both sets of animals, the peripheral blood values were at or above control levels.

With high doses, there is usually a secondary relapse which may result from a decreased blood flow (23,36). This relapse, along with an associated increase in endosteal fibrosis, occurred about 2 months after the local exposure of rats to 2000r (36). In disrupted but unirradiated marrow, blood flow did not return to normal and regeneration was not complete until after 8 weeks (23).

Effects on CFU-S

All blood cells (erythrocytes, white cells, and megakaryocytes) differentiate from a common pluripotent stem cell (CFU-S)

present in the spleen and marrow (16). CFU-S is so designated because when injected as part of a bone marrow transplant into otherwise lethally irradiated mice, this single cell will give rise to a colony of hematopoietic cells appearing as a nodule in the spleen (55). It also homes into the bone marrow and repopulates marrow activity (56). CFU-S is unresponsive to either erythropoietin (57) or colony stimulating factor-spleen (58,59). Erythropoietin stimulates development of erythropoietic cells, while colony stimulating factor-spleen stimulates development of myeloid cells and megakaryocytes (58,59). CFU-S is thought to differentiate into two sets of stem cells; one set committed to myeloid cells and megakaryocytes while another set designated burst forming unit erythroid (BFU-E) gives rise to erythroblast precursors (60). BFU-E gives rise to several colonies of erythropoietic cells, the CFU-erythrocyte precursors (CFU-E), which in turn develop through the proerythroblasts → basophilic erythroblasts → polychromatic erythroblasts to the orthochromatic erythroblasts, which in turn become reticulocytes (60). The proerythroblast through the orthochromic erythroblasts stages are collectively called "normoblasts." The transmit time for normoblasts in the marrow is 5 days, and they are not normally found in the blood. Reticulocytes have a normal marrow transit time of about 2.8 days in the marrow and 1 day in the blood, while erythrocytes have an average lifespan in the blood stream of 120 days (61).

The CFU-S cells comprise about 0.01-1.2% of the cells in the marrow (average 0.6) and about 0.06% in the spleen (16,55,62).

Morphologically, the CFU-S is slightly larger than the small lymphocyte and is distinguishable by the presence of two prominent nucleoli and the absence of the endoplasmic reticulum (62). CFU-S cells are capable of both proliferation and differentiation and under exponential growth rates can double every 22 hours (16,63,64). Most CFU-Ss are normally in the G_0 resting stage (16), although among active cells there are usually as many CFU-S proliferating as are differentiating (64). A key level of stem cells must be on hand before differentiation occurs (35) and below this point, which appears to be between 5-10% (50,65) that of the normal, stem cells are stimulated to proliferate to restore numbers but do not differentiate. During the later portion of hematopoietic recovery from irradiation, the number of differentiating cells increases above the number proliferating (64).

The CFU-S is very radiosensitive and has a D_0 of between 60-162 rads (16,55,63,66-69) and, under hypoxic conditions, a D_0 of about 240 rads (66). It appears from the D_0 data and other evidence that there are at least two subpopulations (70,71), depending on whether the cells are in G_0 resting phase or have been stimulated to carry out DNA synthesis (16,71). The resting population has a D_0 of around 78r (67,70), and the DNA synthesizing population has a D_0 between 113 (63,70) and 160 rads (55,68). Six to 12 months after 500r, nearly twice as many of the CFU-S cells in irradiated animals are in S-phase of the cell cycle, compared to unirradiated controls (35). Cytotoxic drugs administered 2 days prior to irradiation have a radioprotective effect (72,73) which has been postulated to result from the stimulation of the first and resting subpopulation to

convert into the more radioresistant second subpopulation (70). The existence of subpopulations is also supported by density centrifugation of CFU-S which under normal conditions gives three peaks, although there are only two peaks after treatment with vinblastin, a cytotoxic drug used in leukemia chemotherapy (37).

Table 1 summarizes the effects of radiation on CFU-S numbers in mice. Radiation depletion is rapid but recovery is a slow process which increases with increasing dosage (15,18,60).

Like other mammalian cells, CFU-S cells are capable of repairing potentially lethal damage (69,76). When bone marrow cells from irradiated mice are obtained for transplantation, delaying transplantation allows time for repair as reflected by a shoulder on the survival curve without changing the slope (76).

Migratory Stem Cells

One mechanism for repopulation of irradiated marrow is by migration of circulating CFU-S and/or stromal stem cells to the damaged marrow (36,72,77). This has been demonstrated in a number of experiments in which a portion of the bone marrow or the spleen was shielded while the animal received an otherwise lethal dose of radiation (32,42,51,72,78-80). Stem cells from the shielded area migrated and repopulated the destroyed marrow, preventing radiation induced hematopoietic death. A small area of bone marrow destroyed would not be repopulated by this procedure since apparently large areas must be damaged to stimulate this process (81). The migratory stem cell population in the mouse is reported "to be 0.3 percent of

Table 1. Radiation effects on CFU-S.

Dose (rads)	Time post irradiation (days)	CFU-S levels % of normal	Reference
400	1	1-4	16,35
750	1	(1 per spleen)	74
50	1	30	75
	5	40	75
	55	50	75
	60	100	75
150	1	10	75
	25	50	75
	70	70	75
250	1	7	30
1000	1	5	30
2000	1	1.2	30
1000	9 months	80	30
	12 months	75	30

the total CFU population of which 1/3 are in the peripheral blood at any one time" (77). The amount of marrow regeneration following local irradiation is directly related to the population size of the circulating CFUs at the time of irradiation (72). Treatments, such as endotoxin which elevate the circulating CFU numbers when given prior to irradiation result in higher numbers of CFUs in the recovering bone marrow than in the untreated but irradiated controls (72). The importance of migratory stem cells is also shown by aortic anastomoses of irradiated animals with unirradiated animals (82). Rats receiving 1000 rads of Co-60 gamma rays and then anastomosed with unirradiated rats had detectable erythropoietic activity in the marrow within 96 hours while the control irradiated animals did not (82). The recovery of erythropoietic activity was due to deposition of circulating pluripotent stem cells donated from the unirradiated anastomosed partner.

There is a small but normal migration of stem cells from the marrow and spleen to the blood and from the blood to the spleen but migration of stem cells from the blood back to the marrow rarely occurs (37,56,83). Interestingly, cells can migrate into and repopulate a depleted region, and then they or their progency migrate out again (84). This was shown by irradiating the hind leg of a mouse with 2000r, allowing the marrow to repopulate, and then shielding the repopulated leg and irradiating the remainder of the body with 2000r (84). Migratory cells from the repopulated but shielded marrow repopulate the destroyed marrow in other sites of the body.

Stromal stem cells, unlike their hematopoietic counterparts, were thought to be nonmigratory (23,85). Local irradiation of one leg of a mouse sufficient to destroy hematopoietic and stromal cells showed that after 3 days, sufficient numbers of marrow stromal cells had migrated to the damaged marrow to restore their levels to 80% of normal (86). The subsequent repopulation and recovery of the marrow stroma precedes the regeneration of hematopoiesis and illustrates the importance of the marrow microenvironment in regulating erythropoiesis.

Local Repopulation

In contrast to studies which indicate a role for migratory stem cells in the repopulation of radiation depleted marrow, evidence indicates that recovery in certain instances is a local phenomena (16, 85,87,88). These experiments were conducted by using dextran perfusion of the bone marrow to mechanically depopulate the marrow and then monitor its subsequent repopulation (87). The contribution of the local cells in repopulation and regeneration of the bone marrow was aptly illustrated by two experiments. In the first experiment, a mouse received a total body dose of 800 rads of X-rays with the exception of one leg which was shielded (87). Following irradiation, the mouse received a rat marrow transplant sufficient to replace all damaged marrow. The rat marrow was chemically distinguishable from the mouse marrow. The shielded leg was then mechanically depopulated and the marrow regeneration monitored. The cells which repopulated the depleted femur were shown to be of mouse origin, therefore the repopulation was a local event.

In the second experiment conducted with rabbits, one femur was X-rayed and then depleted by dextran perfusion (89). Control animals were depleted in one femur and the rates of repopulation measured. Repopulation was delayed in the femurs which received radiation prior to mechanical depletion, again indicating local repopulation rather than migratory. The radiosensitivity of the regeneration process (based on the number of hematopoietic cells and thymidine uptake by the marrow) was found to have a threshold of 220r, with 325r as the mean effective dose for delaying regeneration (89). The reverse of this experiment was also conducted in which one leg was shielded while the animal was irradiated with the shielded leg subsequently mechanically depleted (90). Irradiation of the surrounding body did not alter the regeneration time in comparison with unirradiated animals who had the marrow of one leg mechanically depleted (90).

Interestingly, mechanical depletion of bone marrow which was aplastic as a result of irradiation (6000r to one leg), followed by a marrow transplant, resulted in regeneration of the aplastic marrow (85). Mechanical depletion or bone marrow transplantation administered separately had no regenerative effect. Bone marrow transplants alone supposedly failed because of the lack of stromal cells to support a hematopoietic environment, where as mechanical disruption failed because of the lack of hematopoietic stem cells previously destroyed by the radiation. Disruption redistributes the stroma. The results tend to indicate that both stromal stem cells and hematopoietic stem cells are not sufficiently migratory to repopulate and regenerate

local damage (23). Damage to larger areas may induce a stimulus for increased migration of these stem cells (35,81). Better marrow regeneration occurred in radiotherapy patients who had large areas of the marrow irradiated compared to those who had small marrow volumes irradiated (81).

Mechanical depletion studies augment histopathological observations which suggest that bone marrow regeneration following local stem cell destruction is initiated from small foci of "new" stem cells migrating outward from the cortex (16,47,91). The "new" cells are thought to arise from the differentiation of previously uncommitted mesenchymal cells in the Haversian canals and endosteum (16,91).

Total Aplasia

Total aplasia of the marrow can result from "1) stromal damage to the vasculature with subsequent fibrosis and 2) irreversible stem cell depletion in the irradiated site" (2). In humans, the marrow regeneration is also dependent on several factors: the age of the patient, the total dose of radiation, and the amount of bone marrow irradiated (17,81). Regeneration is more efficient in younger patients because with progressive age, there is a shrinkage of sites of active bone marrow. By the time humans reach adulthood, active erythropoiesis in the long bones has been predominantly lost, leaving the pelvis and sternum as the major active sites (52). The total dose of radiation is of course important, and 4000 rads locally appears to be the upper limit for marrow regeneration in mice (81). After

6000r to one leg, there was no regeneration and the damaged marrow was replaced by collagen formation and fat deposits (36). Aplasia at these doses appears to be related to vascular damage which makes it impossible to support active marrow growth (36). Similar observations have been made in humans receiving radiotherapy. Estimates of the minimum dose of radiation to a localized area required to produce aplasia in humans has been revised upwards from earlier estimates of 3000r over 3 weeks (93) to a dose above 4000r (94). This is dependent on the volume of marrow irradiated since high doses to small areas are less likely to stimulate regeneration (17,35,81,94). A follow-up study of patients who had received 4000 rads during radiotherapy showed a 40-50% recovery of hematopoiesis in the irradiated marrow beginning between the ninth month to the third year post irradiation (94). In those patients who recovered marrow activity in the irradiated area, the activity was only 60% normal at 12 years post irradiation (94).

Microenvironmental Effects on Bone Marrow Regeneration

Increasing attention has been focused on radiation effects on the microenvironment surrounding the hematopoietic stem cells in turn affecting hematopoiesis (16,17,83,95). The microenvironment can be affected by damage to the stromal stem cells and/or damage to the blood vasculature supporting the marrow (17,33,36,83,95). Its importance in influencing stem cell development is reflected in the ratios of erythropoietic to myelopoietic cells formed from pluripotent stem cells in the spleen and the bone marrow (37,96). The spleen

produces higher ratios of erythropoietic cells, while the marrow produces higher numbers of granulocytic cells (37,96). That this is due to influences from the two organs, rather than differences in the stem cells from these two sources, is apparent from bone marrow transplants to lethally irradiated mice in which the seeded spleen still produces higher numbers of erythrocytes (96). Also, when marrow stroma cells were placed in the spleen, the surrounding spleen stem cells produced more granulocytic cells than erythrocytes, as characteristic of the marrow (96). Damage to the microenvironment may explain why CFU growth rates are similar when control mice and mice which have previously recovered from 950 rads are exposed to 300 rads, although the CFU numbers in the previously irradiated mice plateaued at a lower level (95).

The stromal cells responsible for maintaining the microenvironment are more radioresistant than the hematopoietic stem cells (41, 98). Radiation may indirectly affect the hematopoietic stem cells by directly affecting the stroma. For example, a dose of 500 rads impaired the ability of bone marrow stroma to support bone marrow cultures (97). The _in vitro_ D_o for cells which transfer hematopoietic microenvironment (HMTU, U is for "units") is 444 rads with a theoretical threshold of 500 rads, while _in vivo_ these two values are 325 and 635 rads, respectively (98). This study also estimated that there are about 50 such HMTU cells in a mouse femur, each capable of supporting about 60 pluripotent hematopoietic stem cells (CFU-S). The HMTU has been studied in other labs as the colony forming unit--fibroblast (CFU-F), a stem cell measured it its ability to form stromal

fibroblast colonies capable of hematopoietic support (99,100). The CFU-F has an *in vitro* D_0 of 220r (99) in mice and 200r in guinea pigs (100). Following a dose of 150r, CFU-F levels reach a nadir in mice on about day 4 and return to normal by the twenty-fifth day (99). Reported under the term "marrow stromal colonies (MSC)," cells cultured from mouse femurs irradiated with 1000 rads showed a recovery pattern similar to that seen from hematopoietic marrow cells *in vivo* (34). MSC cell numbers recovered following irradiation but experienced a second depression at 1 year after radiation. Following doses of 5000-10,000r, MSC numbers were only about 10% of the normal value and 1 year later they were still less than 50% of normal. Interestingly, these doses also had abscopal effects on a shielded femur, where the MSC levels in the 5000-10,000r mice were 75% or less compared to normals. Doses of 1000r and greater caused an increase in MSC levels in the shielded femur during the first several days following irradiation, followed by a sharp decrease by 1 month. Radioiron uptake in these animals dropped significantly in the shielded leg as well as the irradiated leg, and was still below normal a year later in those animals receiving 5000r or more. Other abscopal effects have also been reported for blood, and the literature was reviewed by Lawrence in 1948 (39). His own work in which the circulatory systems of irradiated cats were connected to unirradiated cats by carotid anastomoses did not support or demonstrate abscopal effects (39).

The microenvironment is also responsible for controlling the release of mature and stem cells from the hematopoietic organs,

possibly by either a stimulus or by regulation of vascular changes (35,37,101). The CFU levels in protected spleens are reduced within 15 minutes of irradiation, indicating immediate circulatory changes in the microenvironment (35). A migratory factor stimulatory to CFU-S is released within 15 minutes following irradiation and is present in shielded marrow 2 hours after irradiation (91). It causes increased DNA synthesis and proliferation in CFU-S cells but is labile and disappears within 4 hours post irradiation. The levels are dose dependent. Other humoral factors, such as erythropoietin and CFU-S factors, in the microenvironment are capable of regulating higher differentiated stem cells. A number of other compounds with possible effects on the microenvironment, such as serotonin (92), prostaglandins (93), and glycosaminoglycans (94), are known to be elevated following irradiation. Cyclophosphamide given 3 days prior to irradiation enhances recovery of stem cells apparently by affecting the microenvironment, since bone marrow transplants given after irradiation are also enhanced (73).

Macrophages are also important in maintaining the microenvironment, since they secrete humoral factors and remove debris (105). The reticuloendothelial system (RES) apparently removes some damaged stem cells which would otherwise recover, since a carbon-blocked RES allows better recovery of <u>in vitro</u> irradiated bone marrow than do control animals (105).

Radiation Effects on the Spleen

Radiation may have profound effects on the spleen as a result of the radiosensitivity of the hematopoietic stem cells in the germinal centers and because of the ability of the spleen to act in a compensatory fashion for erythropoiesis following severe damage to the hematopoietic tissues (33,34,40). The most comprehensive descriptions were provided by Murray in 1948 (40), and additional data since that time has mainly dealt with the characteristics and properties of the stem cells. Basically, doses below 175r cause negligible damage or change in spleen weight although cellular changes, mainly from the destruction of small lymphocytes, can be seen after a dose as small as 25r (40). The D_0 for producing a change in spleen weight was recently reported for mice to be 196r (67). Doses above 400r generally have the same effects on the spleen and increasing the dose simply delays recovery (40). The upper limt dose for spleen recovery is probably 3000r, with atrophy resulting from destruction of germinal centers and damage to the blood vasculature (106). Therapeutic irradiation of the spleen for malignant lymphoma and for several other conditions requires an average dose of 3900 rads which results in a thickening of the spleen capsule, reduction of the white pulp, and fibrosis of the red pulp and vasculature (107). The organ becomes permanently atrophied.

As summarized by Murray, there are four phases of spleen recovery following irradiation: destruction, removal of debris, inactivity, and regeneration (40). The phases were described from

rabbits receiving 800r ($LD_{50/30}$) but the pattern is similar for most species. Within 30 minutes following irradiation, there is a cessation of all mitotic activity, the death of all small lymphocytes and the majority of stem cells (40). Commensurate with the large cellular destruction and subsequent accumulation of debris, there is a large emmigration of leucocytes and macrophages which begin an immediate phagocytic clean up. This influx causes a brief swelling of the spleen followed in turn by a rapid decrease in spleen weight and size as the debris is removed (19). The clean up process is generally completed within the first day post irradiation, requiring about 8-17 hours following doses of 600-800r (40). The result is a greatly atrophied organ weight perhaps only 40% or less of the original weight (40,51), reduced to mostly a fibrotic structure containing reticular cells and with the germinal centers reduced to small clusters of cells surrounding the arterioles (40,51). Although mitotic activity was completely halted by 30 minutes after irradiation, activity resumes, and regeneration begins about 3 hours after irradiation (40). Both mitotic activity and regeneration proceed slowly during the first 2 weeks. During the inactive period, there is an increase in the fibrotic content (19,40) which will continue if the atrophy persists and regeneration does not occur. After the inactive period, recovery and compensatory mechanisms swing into full gear. The spleen weight increases rapidly, mainly as a result of the increased erythropoiesis, and peaks during the middle to end of the third week post irradiation (33,34,40). Peaking of the spleen weight during recovery does not mean that there are more spleen cells present than in the controls,

but reflects the additional weight from erythropoietic compensation. Mice which received 50, 100, and 500r daily to accumulated total doses of 1000r had peak spleen weights on day 14 post irradiation, although the number of spleen cells did not reach control values until day 28 and were still increasing above control levels on day 60 post (41). The bone marrow cells in these same mice did not return to control levels until day 60 (41).

Repopulation of nucleated cells in the spleen starts later than in the bone marrow (54,108,109). The normal proliferation index (a measure of the DNA synthetic activity reflected by the uptake of ^{125}IUdR averaged over a unit quantity of cells of tissue) (54) of the spleen is about .83% ^{125}IUdR incorporated/100 mg spleen weight (109). This value drops to .1 on the first day post irradiation and peaks on day 13 at about 2.3. The marrow proliferation index is measured as percent ^{125}IUdR incorporated per 10^8 nucleated cells, and increases from a preirradiation value of .55 to 1.8 on day 3, dropping to just above normal by day 15 post irradiation (109). The proliferation indexes indicate that the spleen incurs greater damage, and that the marrow recovers first (108,109). At the same time, however, all of the nucleated cells in the bone marrow are not necessarily proliferating or differentiating stem cells, but may reflect an increase in phagocytic cells infiltrating the marrow. The spleen and marrow also differ in comparison to the degree of cellularity and the proliferation index when the two organs are regenerating following radiation damage. In the marrow, the two values, cellularity and proliferation index, are inversely related while in the spleen they are parallel (109). This may reflect control mechanisms in operation

in the marrow, since there are greater restrictions on available space for cells than those imposed for the spleen. It would seem unlikely that this reflects ineffective cellular control in the spleen, but rather "a shift in set point" to allow for splenic compensation of hematopoiesis during the crises (109). Increasing the splenic erythropoiesis lowers proliferation in the marrow, as does increasing the donor concentration of cells in bone marrow transplants (109).

Variation of the dose rate or fractionation of dose can modify damage. More small lymphocytes were present in the spleen 3 days after a dose of 400 rads when the dose was protracted over 11 hours than when given acutely (110). Lower dose rates and increased periods between fractions can decrease the damage in comparison to an equivalent single acute dose; however, short fractionation periods can increase the damage. Regeneration was slower in rats receiving a divided dose 5 minutes apart than animals which receive a single acute 600 rad dose (111).

II. LITERATURE REVIEW ON ZINC PROTOPORPHYRIN

Introduction

Zinc protoporphyrin (ZPP) (molecular weight 626) is similar to heme except that a zinc atom is chelated to the center of a protoporphyrin IX ring in place of iron (5-8). It is formed both enzymatically (1,2,112) and non-enzymatically (1,2,112,113) and is present in small levels in red-blood cells (5). Humans have about .6 nmol of ZPP per mole of hemoglobin (10,114). The levels of ZPP vary slightly from species to species; rabbits have 2.1 μg ZPP/gram of hemoglobin (115), while mice and humans have 1.9 and 1.2 μg ZPP/g Hb, respectively (10,116,117). Like heme, ZPP may be bound to globin to form hemoglobin (3), although it does not participate in oxygen transport (118). The stable bonding to hemoglobin prevents diffusion out of the cell and hence, once formed, ZPP is retained inside for the lifetime of the red cell (4). Its function in the red cell has not yet been determined, but elevations are observed in several disease states. Since 1974, ZPP has emerged as a valuable diagnostic indicator in human medicine for iron deficiency anemia and lead poisoning (6-8,10,14,15,119,120). Prior to this report, which presents the effects of whole-body irradiation on ZPP, these were the only two conditions known to cause elevations of ZPP levels (3-5).

Biosynthesis of ZPP

Biosynthesis of ZPP and heme proceed by a common pathway, differing only in the metal incorporated to form the end product.

The pathway beginning with the combination of syccinyl CoA and glycine to form δ-aminolevulenic acid is summarized in Figure 1. The first five of the six enzymes in the pathway successively result in the formation of protoporphyrin IX (121,122). The structure of the protoporphyrin end product is identical to that of the ninth porphyrin to have been characterized, hence it was named protoporphyrin IX. The sixth enzyme in the pathway, heme synthetase or ferrochelatase, chelates ferrous iron to protoporphyrin IX forming heme, which then combines with globin to yield hemoglobin. Ferrochelatase is located in the inner wall of the mitochondria (123) and also in the plasma membrane of reticulocytes (124) but is absent in mature erythrocytes. The initial and final steps in the heme synthesis pathway occur in the mitochondria, while intermediate steps occur in the cytoplasm. The first step leading to the formation of δ-aminolevulinic acids is affected by feedback control and is the rate-limiting step.

Presumably, ZPP may be formed *in vivo* either enzymatically and/or nonenzymatically (5). Enzymatically zinc is bound to protoporphyrin IX through the action of a zinc chelatase. Zinc chelatase was first reported about 20 years ago in the bacteria *Rhodopseudomonas spheroides* and also in guinea pigs and rabbits (1). There are conflicting reports concerning whether zinc chelatase is a distinct enzyme or is simply another function of ferrochelatase (2-5,26-31). Some ferrochelatase preparations utilize Zn^{2+} (1,2,112,113,125-127) and some do not (128-130). One report concluded that the two activities belonged to different enzymes (1). This conclusion was reached after finding that the ferrochelatase assay required ascorbic acid and

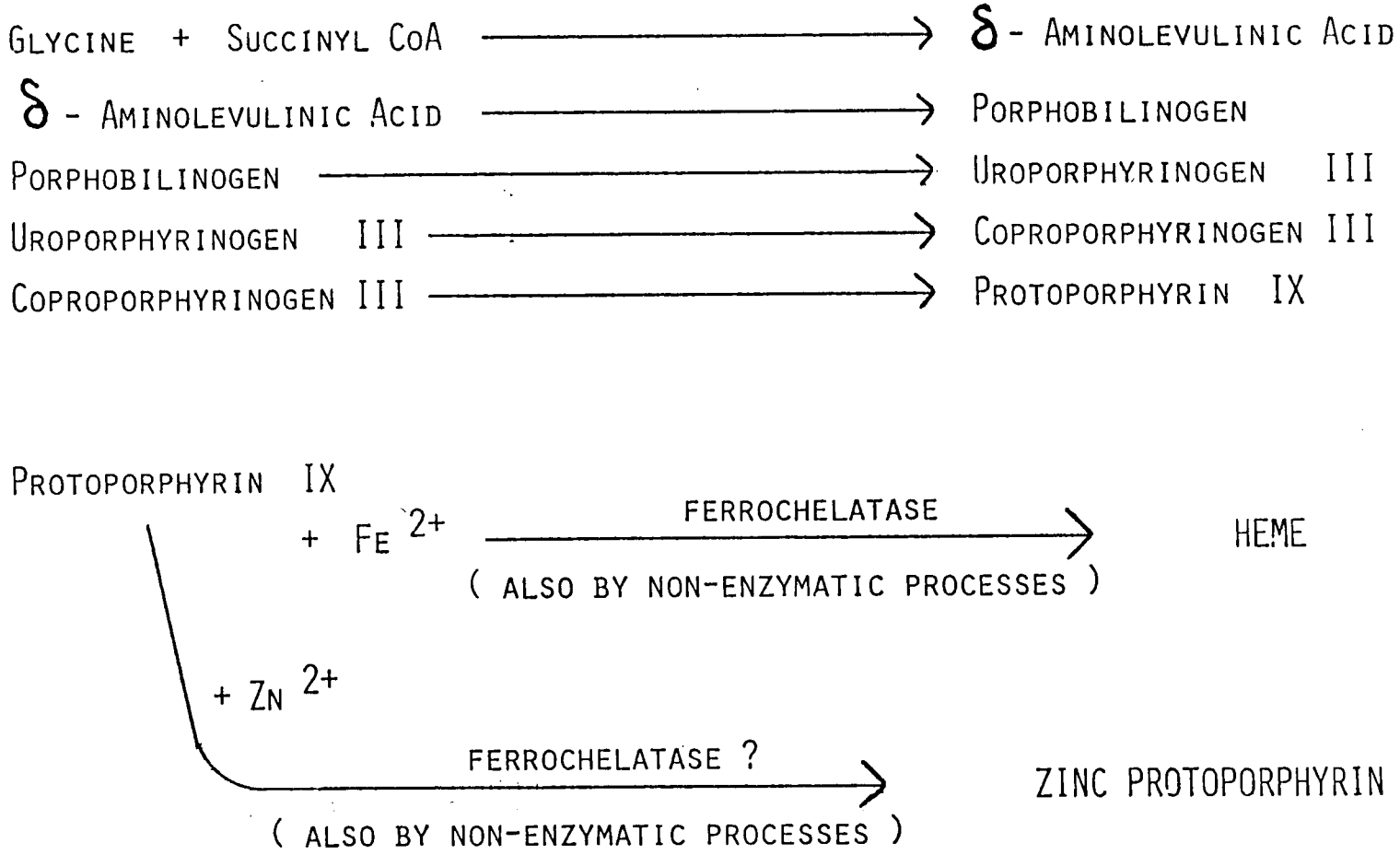


Figure 1. Pathway for heme and ZPP synthesis.

glutathionine, whereas the zinc chelatase required neither and was, in fact, inhibited by glutathionine. Ascorbic acid and/or glutathionine are necessary components in ferrochelatase assys to maintain iron in the plus two valence state which is a specific requirement for enzyme activity. Tween 20 (polyethylene sorbitan monolaurate) plus freezing resulted in loss of zinc chelatase but not ferrochelatase activity. In addition, zinc chelatase was competitively inhibited by Fe^{2+} but Zn^{2+} in turn was noncompetitive for ferrochelatase. After further investigation, the same authors later reported that the two activities, protoporphyrin chelation of iron and of zinc, were most likely carried out by the same enzyme, ferrochelatase (2). The differences in the first paper were attributed to peroxides in the ether which oxidized essential lipid or thiol enzyme groups. As a consequence, "the earlier results . . . are probably explained by the presence of a reducing agent . . . in the ferrochelatase assay but not in the zinc-protoporphyrin chelatase assay: thus the effects of peroxides were eliminated in one assay [ferrochelatase] but not in the other" (2). After retesting with the peroxide-free ether, they concluded that the two chelating activities were the work of the same enzyme. The addition of ether or acetone to the reaction increased the activity of both the zinc chelatase and of the ferrochelatase, presumably by increasing the release of the enzyme from the lipid layers of the mitochondrial membrane or by increasing the solubility of protoporphyrin (2,112). In rat liver mitochondria, both chelatases are located in the inner mitochondrial wall (113). When isolated from barley, both copurify together (126). Complications involving phospholipid

cofactors and loss of enzyme stability prevented the ferrochelatase enzyme from being purified to homogeneity until recently (130). The relationship between zinc chelatase and ferrochelatase has not been examined in the pure preparations; however, zinc was found to be a potent inhibitor of the ferrochelatase enzyme purified from rats (130). It caused 80% inhibition at 10^{-4} M and 99% inhibition at 5×10^{-4} M compared to 0% inhibition produced by the same concentration of $MgCl_2$ (5×10^{-4} M). They did not determine if ZPP was being produced when the zinc "inhibited" the ferrochelatase.

Other evidence that the ferrochelatase enzyme is associated with ZPP formation comes from studies on patients with erythrocyte protoporphyria, a recessive hereditary disease caused by a defective or variant form of ferrochelatase. As a result, heme production is low and protoporphyrin levels are high. In this disease, protoporphyrin exists in the "free" form and not bound to zinc (3). "Free" protoporphyrin leaks out of the red blood cells and accumulates in body tissues where it acts as a photosensitizer, causing skin eruptions and tissue damage in response to sunlight (131). Fibroblasts from patients with erythrocyte protoporphyria, when incubated with zinc, were unable to produce ZPP (132). Patients with porphyria variegata have reduced ferrochelatase activity but normal zinc chelatase activity (132). Fibroblasts from normal patients and those with porphyria variegata were able to form ZPP when incubated with zinc. These experiments show that in erythrocyte protoporphyria there is a variant form of ferrochelatase and also an absence of zinc chelatase activity (132). Several investigators have studied the

possibility of using zinc as a therapeutic agent for the treatment of erythrocyte protoporphyria by converting the "free" protoporphyrin nonenzymatically to the less phototoxic ZPP (131-133). ZPP was formed when an erythrocyte protoporphyrin red cell lysate was incubated with zinc, although therapeutic use in the patient did not yield conclusive results (131). Free protoporphyrin and ZPP levels have also been examined in normal cattle and in cattle which are heterozygous carriers of bovine protoporphyria (134). Free protoporphyrin levels range from 30-70 μg/100 ml of red cells in normal cows and 100-250 μg in the heterozygous protoporphyria carrier cows. ZPP is similarly elevated in the carriers, 80-150 μg ZPP/100 ml of red-blood cells, compared to a range of 50-100 in normal cows.

Elevation of ZPP in Lead Poisoning and in Iron Deficiency Anemia

Prior to our studies with radiation, ZPP was known to be elevated in only two clinical instances, lead poisoning and iron deficiency anemia (3-5). Two enzymes in the heme biosynthetic pathway, δ-aminolevulenic acid dehydratase and ferrochelatase, are inhibited by lead (121,122). δ-aminolevulenic dehydratase is the second enzyme in the heme/ZPP pathway and converts δ-aminolevulenic acid to porphobiligen. Inhibition of the other enzyme, ferrochelatase, leads to elevated levels of protoporphyrin and in turn to elevation of ZPP. Since the enzyme is inhibited, ZPP synthesis proceeds non-enzymatically. ZPP levels would rise since its rate of formation is faster than for the nonenzymatic synthesis of heme. Another

possibility, as pointed out (132) is that perhaps lead inhibition results in an enzyme similar to that in porphyria variegata, where the ferrochelatase activity of the enzyme is diminished but the zinc chelatase activity is normal. Depending on the blood-lead levels, ZPP might be expected to rise in humans from a control value of 1.2 µg ZPP/g Hb to 10-18 µg/g Hb (10). The Center of Disease Control considers levels greater than 18 µg ZPP/g Hb to be extremely high and requiring urgent medical attention (10). This is not a very large portion of the total hemoglobin. At normals levels there are 0.7 nmole of ZPP per mole of hemoglobin (5,10), which a ZPP reading of 18 µg/g Hb only increases the ratio to 1.9 nmole of ZPP per mole of hemoglobin.

The elevation of ZPP following lead poisoning was originally reported as "free" protoporphyrin because the acid extraction procedure removed the zinc (3,5). It was not known at the time that zinc was chelated to the excess protoporphyrin. Free protoporphyrin (FEP) correlated well with blood lead levels in patients with lead poisoning resulting from industrial exposure (13,14) and also in children who became lead poisoned from eating lead-based paints (135). It is also elevated in iron deficiency anemia (136,137). In addition, it was noticed that the blood and urine from lead-poisoned individuals have a higher fluorescent component than samples from normal individuals (138,139). Further comparison of the fluorescent spectra of blood samples from patients with lead poisoning and iron deficiency anemia against that of known standards demonstrated that in these two instances the free protoporphyrin actually existed chelated to zinc (5). The

fluorescent spectra from patients with erythrocytic protoporphyria, a condition in which "free" protoporphyrins are also elevated, indicated that in this condition the "free" protoporphyrins were present in the unchelated state (FEP) (3,5). Studies have shown that in lead poisoning and iron deficiency there is a direct quantitative relationship between FEP and ZPP (6-8,119). This allows a comparison between the two values as determined by the different methods and also allows a reanalysis of earlier data in terms of ZPP. The ZPP level has in turn been found to correlate with the blood lead level in humans according to the equation: log ZPP = 0.023 (blood lead, μg/dl) + 1.04, with a correlation coefficient of 0.90 (120). There was a 0.87 correlation coefficient for the urinary levels of aminolevulenic acid versus ZPP levels (120). In a clinical survey of 111 lead smelter workers, the elevation of ZPP was not correlated to either serum iron, total iron binding capacity, or percent iron saturation and, therefore, the anemia seen in some cases of lead poisoning is not due to an iron deficiency (15). In humans, there appears to be a critical blood lead level or threshold (50 μg/dl) necessary to produce large elevations of ZPP (120). The ZPP threshold is close to the blood lead level of 60 μg/dl determined by the Federal Occupational Safety and Health Agency (OSHA) as a biological threshold for the effects of lead poisoning (140). Using the equation above, health investigators at Bethlehem Steel have calculated that this blood lead level would be equivalent to a ZPP level of 300 μg/dl (120).

The ZPP response seen in humans differs from that observed experimentally in animals (115). In humans, ZPP becomes elevated and may remain so for several months after the blood lead levels return to normal (4). When rabbits were exposed to a single acute injection of lead, the ZPP levels increased from control values of about 2.5 µg/g Hb to 4 µg/g Hb by day 8 and then decreased to normal by day 20 (115). During this same time, there is a sharp rise in free protoporphyrin and, in fact, elevated protoporphyrins in the first peak are predominantly the unchelated form. A second and higher elevation of ZPP forms following the demise of the fist peak. It attains a height of 5 µg/g Hb and, unlike the first peak, does not return to normal levels as rapidly. This peak is predominantly ZPP. The authors attribute the first peak as a response to acute lead poisoning, and the second peak to chronic lead poisoning resulting from residual lead leaking from the tissues (115). They point out that the response in humans resembles the second prolonged peak. The reason for the rapid demise of the first peak is unknown, but it has been hypothesized that it results from leakage of ZPP out of the red blood cell. In rats, both the amount of dietary zinc and the age of the animals have been found to affect the elevation of ZPP during lead poisoning (141). Between ages 30 days to 90 days, the ZPP levels in rats increased from about 1.1 µg ZPP/g Hb to 2.8. Increasing the dietary zinc had no effect on ZPP levels in normal rats but did increase the ZPP levels in lead-poisoned animals up to a point. ZPP levels in humans presumably continue to rise with increasing blood-lead levels, but the ZPP values in the lead-poisoned

rats became elevated and leveled off, although they continued to receive the same amounts of lead in their diet. Interestingly, even though ZPP levels continued to remain high, the rats on lead-supplemented diets had reduced zinc concentrations in the liver and tibia after 140 days on the diet, compared to control animals. There was no significant difference between the lead-poisoned and the control animals in the zinc concentration in the red blood cells but plasma zinc levels were significantly lowered in the lead-poisoned rats.

ZPP also becomes elevated during iron deficiency (5,11,117,142). It is unknown whether the exact mechanism is enzymatic or nonenzymatic, but either case would require a decrease in ferrochelatase activity due to a decreased iron supply. In the enzymatic process, ferrochelatase activity may be decreased while zinc chelatase activity remains (132). ZPP may also become elevated through the nonenzymatic incorporation of zinc (1,2,112,113). In humans, ZPP levels typically reported for iron deficiency are usually lower than for lead poisoning but may reach as high as 10 μg ZPP/g Hb (4,10), compared to normal levels of around 1.2. ZPP measurements have recently been used to screen for iron deficiency anemia in persons who donate blood frequently (117).

Present in this paper is a third condition for the elevation of ZPP as observed in the mouse following a moderate dose of whole-body X-irradiation.

Measurement of ZPP

Zinc protoporphyrins were first reported in 1926 (143), although their detection in biological material was delayed until the 1960's (124) and direct evidence for their presence did not occur until 1974 (5). Procedures in use during this time for the isolation of porphyrins involved extraction into ethyl acetate-acetic acid, followed by extraction with dilute HCl (5,144). The dilute HCl caused the zinc to be split from the protoporphyrin and, as a result, the porphyrin was detected and reported as "free" protoporphyrin (FP) or "free" erythrocyte protoporphyrin (FEP). FEP measured in this manner includes not only the actual "free" or unchelated porphyrin, which is elevated in erythrocyte protoporphyria (EPP), but also the chelated protoporphyrin (ZPP) which was split into the unchelated form by the extraction procedure (5).

ZPP can be made by heating zinc acetate and free protoporphyrin in glacial acetic acid, followed by precipitation with large volumes of water (144). ZPP is readily formed nonenzymatically *in vitro* (1,2,112,113) and presumably *in vivo* (1,2,5,112,113). *In vitro* assays of ZPP formation are depressed by albumin and other proteins present in tissue extracts and lysates which bind zinc and prevent its availability for incorporation into protoporphyrin (145).

Most of the present methods for the extraction of free and zinc protoporphyrins fail in their ability to extract all of the protoporphyrin from the cellular material (3,146) and the degree of efficiency for these procedures varies from extraction to extraction

and lab to lab (1,3,142,146). The most efficient method is the time-honored extraction of free protoporphyrins with ethyl acetate-acetic acid, followed by dilute HCl (3,144). Some procedures are relatively efficient, but the methodology, time, and equipment involved make them unsuitable for clinical assays. For example, one procedure using only 20 µl of blood requires that the sample be extracted in acetone, washed and dried, resuspended with ethanol, then acetone, placed in an ice bath for 20 minutes, centrifuged 10 minutes and decanted, then add propylene glycol and let stand 20 minutes before being read on a fluorometer (134). The procedure is accurate but time consuming.

The two most common methods now in use for the extraction of ZPP are an acetone-water system and an ethanol system. The success of the acetone-water system varies between 10 to 90% of that of the total free protoporphyrins extractable with the ethyl acetate method (3). One lab reported an efficiency of 87% plus/minus a standard deviation of 6.7% with this procedure (146), while another reports that it is reasonably efficient for extracting the free protoporphyrins from EPP (80-90%) (3), although only 10-15% efficient in removing ZPP (12). One modification of the acetone procedure extracts ZPP using acetone containing 0.1 N of NH_3 (1). After the addition of salt this solution is mixed with ether since ZPP is soluble in ether while hemin is not. The procedure was first used to determine ZPP formation in early studies of the zinc-chelatase enzymes and has a reported efficiency of about 60%. The efficiency of the ethanol extraction method varies around 50 to 60%, with one lab reporting an efficiency

of 60% plus/minus a standard deviation of 10% (146). The above methods are not 100% efficient but do provide ways of recovering the ZPP intact. The ethanol procedure was the method of choice used by the first group to identify ZPP as the "free protoporphyrin" elevated in iron deficiency anemia and in lead poisoning (5). A later report by the same authors indicates that the detergent Ammonyx-LO separates ZPP and PP from hemoglobin intact, although the efficiency is not mentioned (3). Prior to the isolation of ZPP from biological materials, procedures had been developed for using a lutidine-ammonia system to separate metalloporphyrins, including ZPP, by paper chromatography (147). This procedure was used to assay ZPP formed by zinc-chelatase in an early paper, but the product was in some way altered and not identifiable as ZPP (1), to my knowledge, and the procedure has not been reported since.

Several methods based on absorption and/or fluorescence spectra are available for detection of ZPP (5,134,146). All porphyrins and their esters and metal complexes readily absorb light at around 400 nm (144). This characteristic absorption band is referred to as the Soret band, which varies slightly for the type of porphyrin and the solvent used (144). In addition to the Soret band, porphyrins may also exhibit other absorption peaks. ZPP in pyridine shows maximum absorption at 589, 550, and 445 nm (1948). Protoporphyrin has a maximum absorption at 502, 537, 576, and 632 nm when measured in a solution of ether-acetic acid, in addition to the characteristic absorption at 395 (the Soret band) (45,50). The molar extinction coefficients for protoporphyrin in ether are 1.35×10^5 at 404 nm,

and 0.94×10^5 for 415 nm (1). The molar extinction coefficients of ZPP in ether at the same wavelength are 0.75×10^5 and 2.40×10^5, respectively (1).

Porphyrins also absorb in the infra-red region, but the most precise method for the detection and quantification of small amounts is by fluorescent spectroscopy. Fluorescence, depending on the choice of solvents and pH, can be used to detect porphyrins as low as 10^{-3} to 10^{-7} µg/ml (144). Free porphyrins emit red light when excited with near ultra-violet light in the region of the Soret band, although in a basic solution this red fluorescence changes to orange (5,134, 144). Protoporphyrin in a dioxane solution exhibits fluorescent emissions of 605, 632, 669, and 704 nm peak wavelengths (144). These change to 604, 626, and 655 nm when protoporphyrin is in 2 N HCl (144). Although free protoporphyrin fluoresces, heme does not (5,144). Only those metalloporphyrins which have a zero magnetic moment fluoresce (150). These include magnesium protoporphyrin, ZPP, and tin^{2+} protoporphyrin, of which only magnesium and zinc complexes are signficant in nature (5). Magnesium protoporphyrin is the basic structural component of chlorophyll (147). When excited at around 425 nm, ZPP will fluoresce with two bands, one at 575-590 nm and a second at 615-620 nm (5). The 575-590 band is shifted to peak at 594 nm when measured in whole blood or in red-blood cells (5). The fluorescent spectra of ZPP is unique and, although there are other compounds with overlapping spectra, the distinction is sufficient that dedicated hematofluorometers have been designed specifically for the detection of ZPP in the whole blood (6-8,114,119). The first

hematofluorometer was designed by the Bell Laboratories but they were not interested in commercial production (8). The instruments now available from several companies are based upon the Bell designs and performance guidelines established by the Center for Disease Control. The earlier instruments excited a sample of blood using 425 nm light (band range 420-430 nm) and measured the emission at 594 nm (580-680 nm) (6,8). At the same that that the sample was being measured, the instrument compared it with an air blank and to an internal standard equivalent to a known amount of ZPP. The machine then reported the value of the sample in μg ZPP/g hemoglobin or as μg ZPP/100 ml of blood. A dedicated hematofluorometer offers several advantages in ZPP determination over the older extraction methods. First, it utilizes one drop of blood in comparison to older procedures requiring as much as 10 ml of blood (6,8,142). Second, time and effort are saved since extraction of ZPP is not required and the entire analysis is completed in about 10-15 seconds. The decreased time of analysis allows more assays to be completed, and some laboratories may do as many as 400 ZPP assays per day (8). Most of the old extraction procedures are at best 80% effective, and so measurements from laboratory to laboratory may vary depending on technical skill and experience. The hematofluorometer eliminates this problem since the assay is automated. The operator need only put a drop of blood on a coverslip, place the coverslip in the detector, and push a button. The results are highly accurate and reproducible; and the machine is portable which makes it convenient for field use.

Sources of Error in Measurement by Hematofluorometer

Several possible sources of error in ZPP determinations made by dedicated hematofluorometers have come to light as a result of the increasing popularity and usage of these machines (151,152). These sources stem from both a design characteristic and from the minor overlapping of fluorescent spectra of components normally found in serum. The design characteristic "front surface illumination" is a special feature of the machine which allows ZPP readings to be made independently of hemoglobin concentration or of hematocrit (6,8,151,152). In detection by this method, the sample of blood is placed on a microscope coverslip and placed in the detector's sample slot where it is carried into the detector. The filtered monochromatic light source used to excite the ZPP is angled up through the glass coverslip and absorbed into the blood sample. The light which fluoresces from the sample then passes back through the glass where it is measured by a detector set at a similar angle from the center of the sample as the exciting light source. This design differs from the normal laboratory spectrophotometer in which light is transmitted through the sample and detected on the opposite side, a 180° travel and detection. In the case of the spectrophotometer, samples which are too concentrated and therefore absorb too much of the transmitted light cannot be accurately measured and must be diluted down into an absorbance range which the machine can quantitatively detect with accuracy. An undiluted sample of blood would be beyond this range. Fluorescence used to detect much smaller concentrations would be

swamped if light traveled through large sample distances. A large sample would naturally fluoresce so much light that it would be out of the accurate operating range of the detector and, as a result, would also have to be diluted. The "front surface illuminator" detection eliminates sample size requirements since only blood components in contact with the glass at the sample window can participate in the ZPP determination. Those components, primarily hemoglobin, would absorb the exciting light and block its transmission very far beyond the first several layers of cells and quench transmission of the fluorescence from these same cells back to the detector. Since the machine determines ZPP proportional to the amount of hemoglobin, a sample of 20 µl or 200 µl of blood with have the same ZPP/Hb ratio (6,8,151,152).

The first source of error that arises in ZPP determination is a time factor (151,152). Since the machine measures fluorescence at the front surface, serum components which fluoresce and are located at the front surface will cause false elevations in the ZPP value. With increasing time following the placement of the sample on the coverslip, more red blood cells will sediment to the bottom or front surface, displacing the serum and lowering the false elevation (152). The ZPP value will level off when a uniform layer of cells has sedimented across the front surface. The average time required for this to occur is reported to be about 5 minutes (152). We found the equilibrium time to be about 6 minutes, in good agreement with the reported value. In most cases, 70-80% of the ZPP value decay occurred in the first minute and was complete by the second

minute, but was in every case complete at 6 minutes. This source of error is not very noticeable at normal hematocrit ranges, but is for low hematocrits (151,152). The hematofluorometer itself will read the provided standards with plus or minus 5% accuracy (114).

Another source of error is attributable to increases in the levels of serum components whose fluorescent spectra overlap that of ZPP (151,152). The most important of these is bilirubin, an intermediate in the breakdown of porphyrins. Free bilirubin, unbound to serum proteins or to red blood cells, will absorb light in the range of 420-430 nm, the excitation wavelength band used by the hematofluorometer, at 50 to 60% of its maximum abosrption which occurs at 465 nm (153). When excited at 440 nm with a band of 10 nm, the emission spectra starts at 480 nm and peaks at 530 nm. Almost 30% of the relative intensity is still present at 600 nm. Although free bilirubin is a weak fluorescer, it does not usually exist in the serum in a free state, but rather bound to albumin. The binding of bilirubin to albumin causes an enhancement in the intensity of the bilirubin fluoresence by about a factor of 10 (154). The peak absorption of the bilirubin-albumin complex occurs at 440 nm and the complex has an emission maximum at 530 nm when excited at 460 nm. The relative intensity of the fluorescence drops from 60% to 35% between 560-580 nm following excitation at 460 nm. The intensity of the remainder of the bilirubin-albumin spectra which might overlap into the ZPP detection range was not given. The effects of bilirubin concentration on ZPP determination by dedicated hematofluorometers have been investigated and it was shown that for every mg of

bilirubin/100 ml of blood in the sample, the ZPP measurement increased falsely between 25 to 40 µg of ZPP/100 ml of red blood cells (151). Using the reported hematocrit value of 35% and assuming 14 g for hemoglobin/100 ml of blood, this value converts to an increase of between 0.6 to 10 µg of ZPP increase for each mg/100 ml of blood increase in bilirubin when ZPP is measured in g ZPP/g Hb, as is the case for the ZPP meter used in this investigation. In our laboratory we adjusted the bilirubin concentration of a human blood sample (hematocrit 50) to 20 mg/dl which in turn increased the ZPP from 2.0 µg ZPP/g Hb to an initial increased reading of 15 µg ZPP/g Hb. After 6 minutes, this value had leveled off to about 8 µg ZPP/ g Hb. The initial reading represents a false increase of about 0.7 µg ZPP/g Hb for each mg/dl blood increase in bilirubin. The actual increase measured at the time of leveling off (6 minutes) is 0.3 µg ZPP/g Hb false increase per mg/dl of blood increase in bilirubin. Since the authors in the other study read their samples only once with an immediate reading not allowing the sample to level off (151), our value which is half their reported value is more accurate. In order to entirely remove the effects of bilirubin, the red blood cells must be washed in saline to remove bilirubin and then resuspended in saline for measurement (151). Removing the serum is not sufficient since the bilirubin may also be bound to the red blood cell. In any case, the manufacturers have built a correction factor into the hematofluorometer for the normal bilirubin ranges in human blood (6,8). The total bilirubin levels in humans normally range from 0.2 to 1.0 or 1.5 mg/dl of blood (155), while the range in mice is

0.1 to 0.4 mg/dl of blood, with an average level determined from 27 mice as 0.31 mg bilirubin/dl of blood.

Decreasing the hematocrit, another source of false elevations in ZPP measurements, is equivalent to increasing the serum fluorescent components (151), particularly bilirubin. Unlike a true serum increase of bilirubin where there is increasing binding of bilirubin to the red cells and an increase in the contribution of small amounts of serum in contact with the front surface, false elevation caused by hematocrit changes may be eliminated by following the ZPP decay to the equilibrium or leveling off point. Since the ZPP response to hematocrit results from fluorescent serum components, hematocrit has no effect when washed cells are suspended in saline for measurement (151,152). In a similar manner, hemoglobin was found to affect the ZPP reading only to the extent that it reflected a decrease in the hematocrit (151).

Prior to its identification in red cells in 1974, ZPP had been observed and identified in the literature as free erythrocyte protoporphyrin (FEP) (121,135-137,142). Erythrocyte porphyrins were known to become elevated following lead poisoning (121,135), iron deficiency (136,137,142), and in porphyria cases (5). They were isolated by acid extraction procedures which removed the zinc, leaving free protoporphyrin (5,144). Free protoporphyrin in whole blood has a fluorescent emission peak of 625 nm when excited at 403 nm (5). The ZPP emission spectra has a maximum emission peak at 594 nm when excited at 420, and it is not appreciably affected by FEP overlap

(5,146). ZPP, on the other hand, has a smaller second peak emission at 640 nm which can overlap on FEP fluorescence in whole blood (146,156).

A modified hematofluorometer has been successfully used to monitor free protoporphyrin and ZPP levels in mice with congenital hemolytic anemia (156). Free protoporphyrin is the predominant form in these mice. Measurements made with the ZPP meter were compared to values obrained by an extraction procedure. The ZPP meter, because of its discriminative ability for ZPP (narrower window), was found not to be useful for measuring free protoporphyrins since the values determined by the hematofluorometer were less than those obtained by the extraction procedure. A modified hematofluorometer containing a larger window capable of receiving both ZPP and free protoporphyrin did agree with the results obtained by the extraction procedure. The earlier models of hematofluorometers described in the literature have windows large enough to detect ZPP and free protoporphyrin (6,8).

Biological Effects of ZPP

The biological consequences of elevated levels of zinc protoporphyrin are for the most part unknown. It is known that the ZPP-globin complex is unable to transport oxygen, probably related to the dimagnetic property of ZPP (118). Although unable to transport oxygen, Zn^{2+} and ZPP do effect respiration activity (157-160). Zn^{2+} causes a shift to the left in the oxygen dissociation curves of relative oxygenated hemoglobin versus the partial pressure of oxygen

measured in nm of mercury (157). Human red blood cells incubated in a solution of 1.5×10^{-3} M $ZnCl_2$ caused a shift of 1.5-3.5 nm of mercury, with a mean of 2.5 nm in the measurement of the p50 (the partial pressure of oxygen at which the hemoglobin is 50% saturated). As a result, there is some hope that the oral administration of zinc may be used to treat sickle cell disease where a decrease in the p50 will allow the sickle cell to pass out of the capillary before becoming sickled (157). The shift to the left indicates an increase O_2 affinity and a decreased O_2 release. The data indicates that this is not due to ZPP but rather to Zn^{2+} binding to an amino acid site and altering stability of the oxygenated hemoglobin (158). ZPP is also found in yeast grown on a zinc supplemented diet where it was noted to be inhibitory to the biosynthesis of succinate: cytochrome c reductase and $NADH_2$:cytochrome c reductase, both of which would normally occur in the presence of oxygen (159). The cytochrome content and oxygen consumption were reduced in comparison to yeast grown on a non-zinc supplemented diet. The work for yeast contrasts work by the same authors on the effects of ZPP on rat liver homogenates, where they found an 18.6% increase in oxygen consumption (160). The methyl protoporphyrin ester had no effect.

Protein synthesis in the red cell is linked to the production of hemin through the hemin-controlled translational repressor (HCR) (161). In the presence of hemin, the HCR exists in the cell in an inactive state, becoming active in the absence of hemin, phosphorylating and activating other HCRs. Activated HCR phosphorylates and inactivates protein elongation initiating factor 2 (eIF-2) and, as a result,

halts protein synthesis. Metalloporphyrins can control protein synthesis by inhibiting the HCR and stimulating protein synthesis (161). Protoporphyrin is about 60% as effective as an inhibitor of the HCR as hemin although, unlike hemin, it is unable to stimulate protein synthesis. Measured in a cell-free or lysate system, ZPP is as effective as hemin in sustaining protein synthesis (162,163). ZPP does not work as well in the intact cell (162). In the intact cell, if Fe^{2+} at a concentration of 2×10^{-4} M is assigned a relative protein synthesis unit of 1.0, in comparison, hemin at a concentration of 1×10^{-4}M will have a relative protein synthesis of 1.10 units. ZPP measured in concentrations of units of absorbance at 399 nm per ml at 11, 22, and 33 A_{399} units/ml will only promote protein synthesis of 0.33, 0.34, and 0.41 relative units, respectively (162). Differences in the effects of ZPP in the cell-free system and in the intact cell are not at present understood (162).

One beneficial effect of ZPP in conditions in which it becomes elevated is to render non-toxic the free protoporphyrin which would otherwise become elevated (5,131). The photosensitizing skin eruptions and tissue damage seen in FEP are not present in lead poisoning or iron deficiency anemia because excess free protoporphyrin has been converted to ZPP. Although the mechanisms for the phototoxic effects of free protoporphyrin are not fully understood, it has been proposed that the ZPP is less phototoxic because of its inability to produce singlet oxygen (162). Oral zinc therapy has been administered to erythrocytic protoporphyria patients in attempts to convert FEP to ZPP, but the results are inconclusive (131,133).

ZPP injected subcutaneiously in a concentration of 50 μmole/kg to rats caused a 50% decrease in hepatic heme oxygenase activity and a corresponding 44% decrease in δ-aminolevulinate synthetase activity when rats were killed and assayed for the enzymes 16 hours later (118). Heme oxygenase is responsible for converting heme to bilirubin. The ZPP caused an increase in the K_m of bilirubin and is also a competitive inhibitor of heme oxygenase (118,165). Unlike heme, ZPP is not degraded by the enzyme because of the inability of ZPP to bind oxygen. The authors were screening for agents which would lower bilirubin levels and prevent the kernicterus caused by high bilirubin levels in neonatal jaundice (118,166). They opted for tin protoporphyrin over ZPP because the tin protoporphyrin did not inhibit δ-aminolevulinate synthetase activity. ZPP administered 100 mole/kg at birth, 12, 24, 48, and 72 hours later (total dose 500 mole/kg) caused a decrease in serum bilirubin levels 5 to 10 days later (166). Tin protoporphyrin caused bilirubin levels to decrease the same day it was administered and required doses 50 times less concentrated than those required for ZPP (166). While ZPP is a naturally occurring substance, the authors did not feel that the amounts of ZPP leaking from red cells during lead poisoning or iron deficiency anemia were sufficient to elicit a reduction of serum bilirubin *in vivo* (166).

Leakage of ZPP From Red-Blood Cells

A simple *in vitro* experiment showed that free or unchelated protoporphyrin leaks from red cells at appreciable rates (4) while ZPP does not. Erythrocyte protoporphyria cells (FEP is unchelated) and cells from patients with lead poisoning and iron deficiency anemia were incubated in plasma and leakage rates of ZPP and FEP were determined. The authors reported that .1% of the ZPP leaked out from the lead poisoned and iron deficiency anemia cells per hour compared to .3 to .7% of the total free protoporphyrin per hour in the erythrocyte protoporphyria patients. When cells were examined by age, it was found that the older cells still retained most of their ZPP content. ZPP appears to remain inside the red cell for the cell's lifetime with decreases simply reflecting a loss of cells with high ZPP content (4). The first peak elevation of ZPP following acute injection of lead into rabbits has doubled in ZPP levels and then returned to normal in the space of 20 days (115). During this time the hematocrit stayed fairly constant. Our radiation-induced elevation of ZPP increased and decreased in a similar amount of time. The leakage rate or disappearnace of ZPP appears to be tied to whether the inducting effect was acute or chronic.

III. METHODS AND MATERIALS

Materials

Erythropoietin, step I, was obtained from the Connaught Medical Research Lab, Toronto, Canada. Mesoporphyrin IX was obtained from Porphyrin Products, Logan, Utah. WR-2721 [S-2-(3-aminopropyl-amino)-ethylphosphorothioc acid] was a gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. [^{59}Fe], 11.8 mCi/mg was obtained as ferrous citrate in saline from ICN. Serum iron, total iron binding capacity and unbound capacity, were determined using the [^{59}Fe] Serum Iron/Iron Binding Capacity Radioassay Kit from Corning Medical and Scientific (167). Protein was determined by the method of Bradford (168) with a kit from Bio-Rad. Total serum bilirubin was determined by the direct spectrophotometric method of Meites and Hogg (169). Hemoglobin concentrations were measured using a Hemoglobinometer (Coulter Electronics), while red-blood cells were counted by use of a Coulter Counter.

Methods

X-irradiation of Mice

Mice were irradiated with 250 KVP X-rays at 15 mAmps using a G.E. Maximar 250 III unit with a 1.0 mm aluminum filter and a target to object distance of 50 cm. The average exposure rate was approximately 80 Roentgens per minute (2.06×10^{-2} coulomb/kilogram/min).

In most experiments, the standard dose was 550 rads (5.5 grays). Mice were placed in a circular plastic pan having 10 equal compartments which was rotated under the beam to insure equal exposure. Unless mentioned otherwise, the mice used in these experiments were C3H/HEJ males, 6 to 8 weeks olds, weighing 16 to 24 grams. They were housed 10 or less to a cage and fed and watered ad libitum.

Zinc Protoporphyrin (ZPP) Measurement

ZPP levels were determined using a dedicated hematofluorometer (ZPP Meter™, Aviv Associates). Samples of whole blood collected in a heparinized microcapillary tube were placed on a Corning brand 25 x 25 mm glass coverslip. One drop of blood (about 25 µl) was sufficient for an assay. The coverslip was inserted into the detector and excited at 420 nm, and the fluorescence of the sample at 594 nm was measured. The data is usually expressed as µg ZPP per g hemoglobin. The readings were taken 10 minutes after the blood was placed on the coverslip. This time interval allowed a uniform layer of red blood cells to settle on the coverslip, thereby eliminating errors due to differences in hematocrit between samples (151,152). The samples were assayed four times and the average was recorded.

Treatment with WR-2721 [S-2-(3-aminopropylamino)-ethylphosphorthioic acid]

The radioprotectant drug (WR-2721) was dissolved in distilled water and administered intraperitoneally to mice 15 minutes prior to

irradiation in accordance with the procedures of Yuhas (170). The dosage given was 438 mg/kg. Fifteen minutes after injection, the mice received 550 rads of 250 KVP X-rays.

Determination of Serum Iron, Total Iron Binding Capacity, and Unbound Iron Binding Capacity

Forty mice received 550 rads of X-rays and were killed in groups of five on the indicated days after irradiation. Five unirradiated animals served as controls. The mice was anesthetized; blood samples for ZPP determination and hematocrit determination were taken in microcapillary tubes from the orbital sinus. The mice were then exsanguinated through the left brachial artery using a heparinized pipette. The blood was centrifuged, the plasma recovered and frozen at -80° until assayed. The plasma iron, the total iron binding capacity (TIBC), and the unbound iron binding capacity (UIBC) were determined using the Corning Serum Iron, Iron Binding Capacity [^{59}Fe] Radioassay Kit (167). Two 50 µl samples of plasma were taken for each assay. One sample was used for the serum iron determination and the other for the UIBC determination. The amount of [^{59}Fe] retained by the resin during the assay was determined using a gamma counter. The TIBC was calculated from the formula: TIBC = UIBC + Serum Iron. Three different standards with known TIBC, UIBC, and serum iron levels provided with the kit were used to standardize the results. All three--TIBC, UIBC, and plasma iron--were reported in µg iron/dl of serum. A fourth value useful in diagnosing iron deficiency, the percent iron saturation, was

calculated by dividing the plasma iron level by the TIBC and multiplying the result by 100. The percent iron saturation indicates the portion of the TIBC that is being used to carry iron in the plasma. An iron saturation value below 16 is indicative of iron deficiency (171).

Splenectomy

Splenectomies were performed according to Layendecker (172) 1 week prior to irradiating mice with 550 rads. The wound clips were removed just prior to irradiation.

Ferrochelatase Assay

Ferrochelatase was assayed by using a modification of the method of Williams et al. (173). Livers and spleens were excised from the mice on the indicated days after irradiation and also from the unirradiated controls. Five ml of homogenization buffer (0.25 M sucrose, 0.05 M Tris, pH 7.5, and 0.01% Tween 20) were added to 5 grams of spleen or 5 grams of liver. The tissues were then homogenized and sonicated at high speed for 30 seconds with a Polytron homogenizer (Brinkman Instruments), then cooled with ice for 1 minute. The homogenization-sonication was repeated for 30 seconds. Samples of blood from irradiated and control animals were prepared by pooling 1 ml of heparinized blood from each animal (minimum of 10 animals) and then removing a 5 ml aliquot from each pooled group. Each 5 ml aliquot of blood was mixed with 1 ml of the homogenization buffer and subjected to the same procedures described for the livers and spleens. The protein concentration of each of the homogenates

(liver, spleen, and blood) was determined and the samples were diluted with homogenization buffer to a concentration of 50 mg protein/ml.

Stock solutions for the assay were made up according to Williams et al. (173) with the exception of the "working" iron solution. The "working" iron solution contained 10 mg ascorbic acid, 8.35 mg $FeSO_4$-$7H_2O$, 10 mg reduced glutathione, and 7.44 μCi of [^{59}Fe] as ferrous citrate (specific activity 11.8 mCi/mg), in 3.0 ml of deoxygenated water. The working iron solution also contained 62,000 cpm of [^{59}Fe] per 25 μl (the amount added to each reaction tube). Further modifications were made to reduce the amount of sample in each assay tube. Each reaction tube contained 50 μl of Tween 20, 250 μl mesoprophyrin IX (42.5 nmole in 95% methanol), 1.25 ml of 0.3 M Tris, pH 7.5, and 250 μl of homogenate. Plastic culture tubes with caps were used for reaction vials. The reaction mixture, minus the working iron solution, was flushed with N_2 and capped. The tubes were incubated at 37° for 5 minutes, after which 25 μl of the working iron solution was added and each tube again flushed with N_2 before recapping. After incubating the tubes at 37° for 25 minutes, carrier heme 125 μg was added, and the total heme extracted with ethyl acetate/glacial acetic acid as described by Williams et al. (173). The incorporation of Fe^{2+} into heme was linear up to 45 minutes. Samples were counted in a gamma counter.

Studies on the Combined Effects of X-irradiation and Lead

Lead was administered to 38 mice by placing lead acetate in their drinking water at a concentration of 4×10^{-3} M for 4 weeks. For the next 18 days, the lead acetate concentration was increased to 5×10^{-2} M. The degree of lead poisoning was monitored by measuring the ZPP and by assaying blood lead levels via atomic absorption spectroscopy. After 30 of the lead-poisoned mice had been irradiated with 550 rads of X-rays, they were divided into two groups of 15. The first group was continued on lead acetate (5×10^{-2} M) while the second group received normal water. The mice were monitored for ZPP levels and hematocrit with no more than 50 µl of blood being taken from each mouse per week.

IV. RESULTS AND DISCUSSION

Results

Elevation of ZPP After Irradiation

Unless otherwise stated, the experiments described below were done with C3H/HEJ male mice which received 550 rads. The blood ZPP level was determined at the indicated time post irradiation as shown in Figure 2. The average ZPP level of unirradiated C3H/HEJ mice (n = 67) was 1.9 µg ZPP/g Hb and the average hematocrit was 45. Similar ZPP levels were also found for a different strain, CD-1 Swiss outbred (Charles River Co., Wilmington, MA), and also for Sprague-Dawley rats. There was no difference in the normal ZPP levels between males and females.

Following whole-body irradiation, ZPP levels remained constant at about 1.9 µg ZPP/g Hb through day 7 when they rose slowly through day 16 to 2.8. At day 16, the ZPP level rose rapidly, reaching a peak of 4.3 on day 20 post irradiation. The ZPP then decreased at about the same rate with which it had increased. ZPP levels returned to normal by day 35.

Also shown in Figure 2 are the hematocrit values which decreased on day 7 and dropped to the lowest point on day 13 or 14. The sharp rise in ZPP (days 17-21) occurred at the same time that the hematocrit rose (days 17-20).

Effect of Radiation Dose on ZPP Elevation

The elevation of ZPP was not observed at doses of 300 and 400 rads but was seen at a dose of 450 rads. After receiving 400 rads,

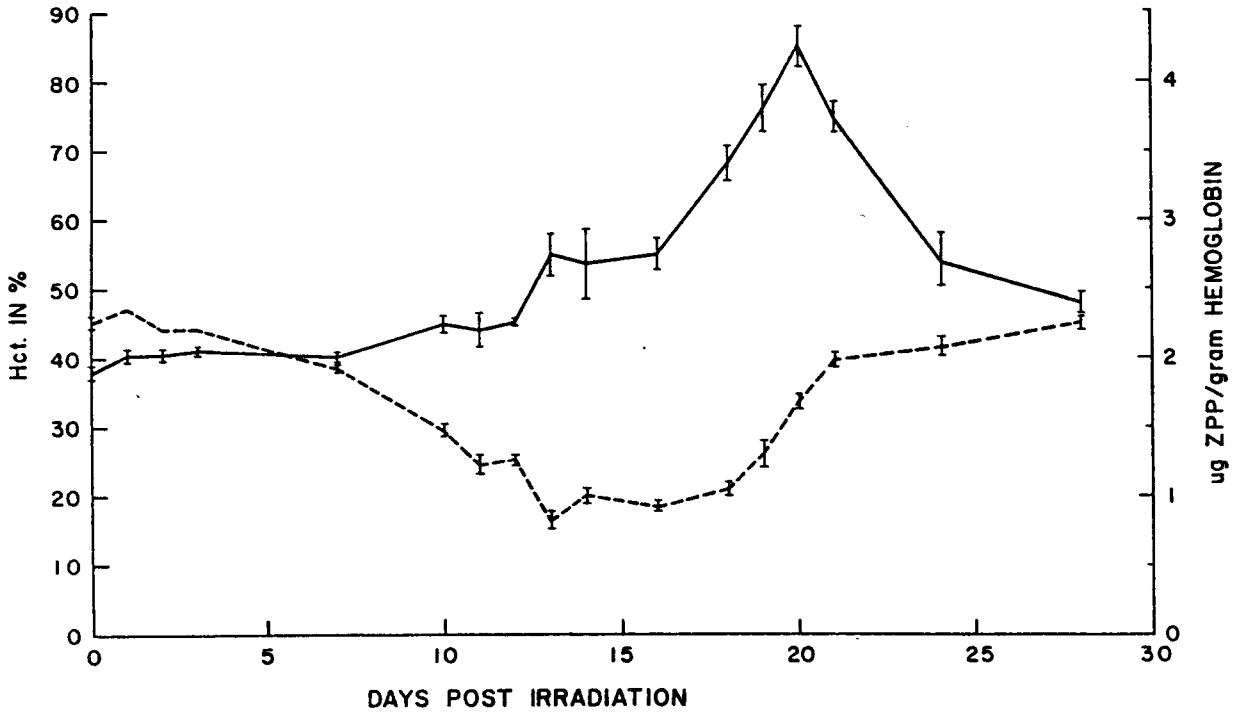


Fig. 2. Elevation of blood ZPP levels following whole-body X-irradiation.

C3H/HEJ male mice received 550 rads of 250 KVP X-rays. On the indicated days the hematocrit (---) and ZPP (——) concentration were measured. On days 5-16, there were 20 mice per data point and on days 18-24 there were at least 5 mice per point. The control value, day 0, is the average of 67 mice. Standard error bars for each data point are provided.

mice were examined for ZPP every 2 days (5-10 mice per data point) up to day 24 post irradiation with no apparent change in ZPP levels from those of unirradiated controls (not shown). This finding indicates the existence of a threshold below which radiation does not induce ZPP elevation. The elevation of ZPP was not observed in C3H/HEJ mice or rats exposed to lethal X-ray doses.

Having determined the lower limit below which ZPP was not elevated and the upper limit set by hematopoietic death, we focused our attention on the effect of radiation dose between these limits. As shown in Figure 3, when mice received 450, 500, or 600 rads of X-rays, the ZPP rose to the same level regardless of dose, but the onset of the elevation of ZPP was delayed at the higher doses of radiation. In a different strain of mice, CD-1, the threshold below which ZPP elevation did not occur was 600 rather than 450 rads. In rats, the threshold was also 600 rads.

Comparison of Serum Bilirubin and ZPP Levels Following Irradiation

Bilirubin absorbs at 420 nm, and its fluorescent emission slightly overlaps that of ZPP (151,152). The fluorometer used in these studies has a much greater sensitivity for detecting ZPP than for bilirubin since its window is set on the ZPP emission peak; at this wavelength there is very little overlap by the emission from bilirubin.

However, we felt that it was necessary to eliminate the possibility that the elevation of ZPP was a spurious result which

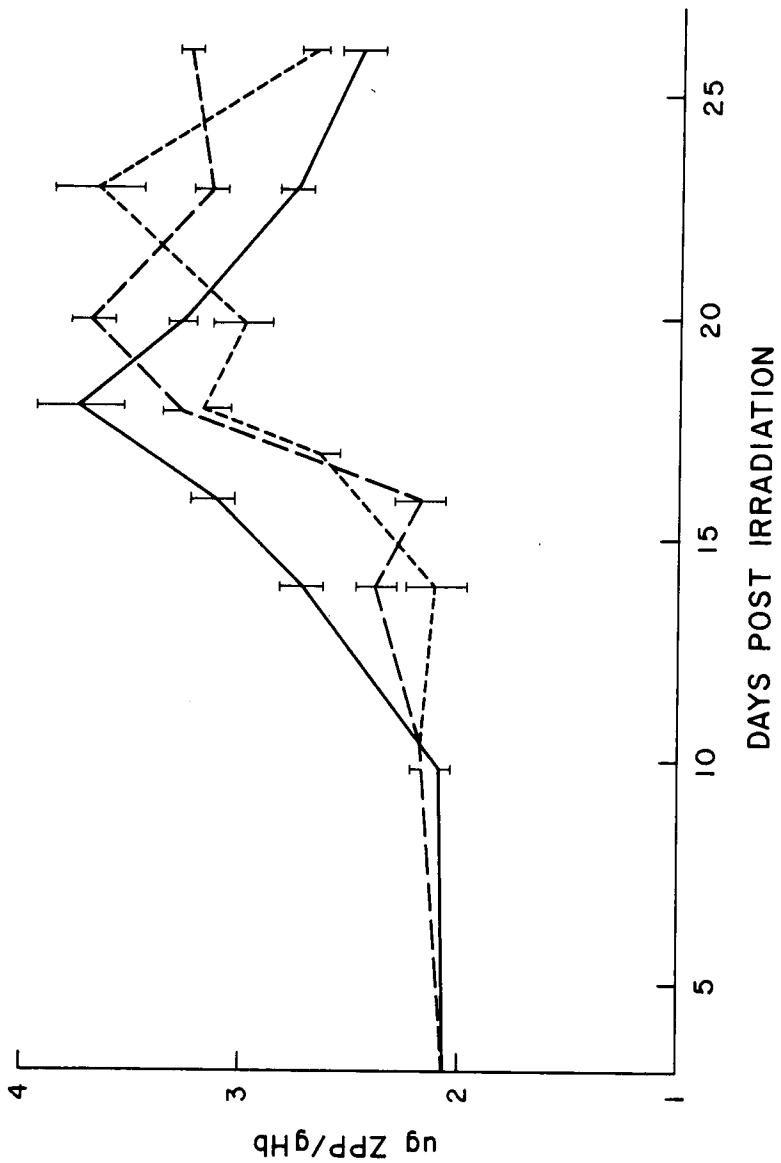


Figure 3. Increasing the radiation dose delays the onset of ZPP elevation.

C3H/HEJ male mice were irradiated with 450 (——), 500 (— —), and 600 (---) rads of X-rays and the ZPP determined on the indicated days after irradiation. Each data point is an average from 5 mice. Standard error bars are provided.

was actually due to bilirubin elevation. To rule out this possibility, 40 mice received 550 rads of X-rays and were then killed, 5 each at days 1, 2, 3, 7, 10, 14, 16, and 21 post irradiation. Twenty-eight unirradiated mice were used as controls. The mice were anesthetized and exsanguinated using heparinized pipettes, and hematocrit and ZPP determinations were made for each mouse. The blood was then centrifuged and the serum collected. The serum bilirubin concentration was then determined from the abosrbances at 455 and 575 nm using the direct spectroscopic method of Meites and Hogg (169). The normal bilirubin level in C3H/HEJ male mice, as determined from the 28 unirradiated controls, was 0.30 mg/dl.

The change in serum bilirubin levels following irradiation and the ZPP concentration during the same period are shown in Figure 4. Bilirubin levels approached zero with an average of 0.15 mg/dl on day 2 and remained low through day 7. These levels returned to slightly above normal by day 10 and continued to rise, reaching a peak of 1.06 mg/dl on day 16 before decreasing to normal levels by day 21. The elevation of ZPP could not be a spurious consequence of bilirubin elevation since ZPP was still elevated on day 21 at which time bilirubin has dropped to normal. We confirmed these results in yet another way: the red cells were washed with saline to remove bilirubin and the ZPP continued to remain elevated (data not shown).

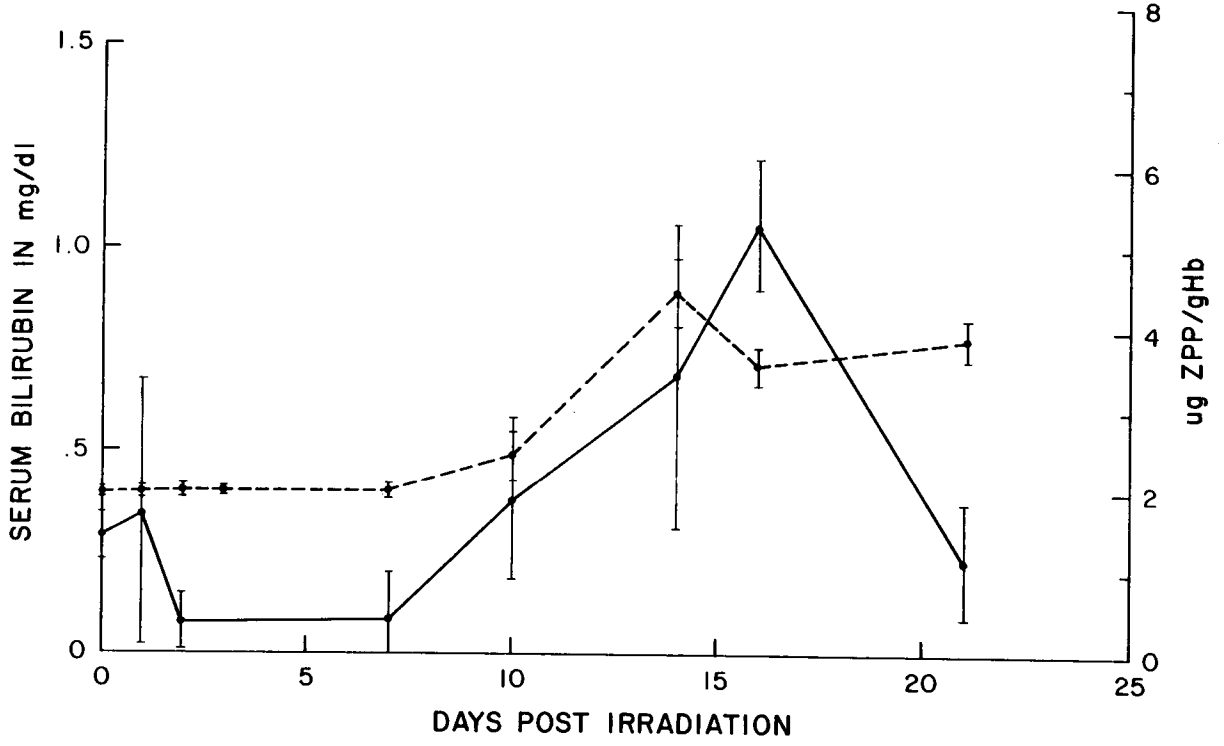


Figure 4. The radiation-induced elevation of ZPP is not attributable to bilirubin.

C3H/HEJ male mice received 500 rads of X-rays. Serum bilirubin (——) and ZPP (---) levels were measured on the indicated days post irradiation. Each data point represents an average of 5 mice.

Hematologic Parameters Following Irradiation with 550 Rads of X-rays

Various hematologic parameters were compared with ZPP values at the indicated times after irradiation (Table 2). Initially, the ZPP slowly increased while the red-cell count, hematocrit, and hemoglobin concentration decreased. After 16 days, these parameters began to rise. On about day 14, the ZPP level began to increase. The ZPP concentration per red cell can be calculated from the MCH which expresses the average hemoglobin concentration (in pgms) per red cell. This value for unirradiated mice was approximately 34×10^{-18} grams of ZPP per RBC. The values for days 16, 19, and 21 post irradiation were 57, 85, and 90×10^{-18} grams ZPP/RBC, respectively. The value of red blood cells (MCV) decreased until day 16 when the MCV then increased to a value of 73 μm^3 by day 21. These high MCVs are indicative of a population of newly synthesized red cells (young red cells have larger volumes than older cells), and the increase in ZPP concentration per cell indicated that the elevation of ZPP at 16 days post irradiation was due to elevation ZPP levels in young newly synthesized cells as the hematopoietic system recovered from radiation damage. Between days 12 to 24 post irradiation, the red cell count increased from 5.18×10^6 RBC/mm^3 to 7.44×10^6, with a corresponding decrease in ZPP from 3.7 to 2.4 µg ZPP/g Hb. The sharp decrease in ZPP following day 20 might be the result of diluting out high ZPP-containing red cells by cells containing lower amounts of ZPP.

Table 2. Hematological parameters in C3H/HEJ male mice after receiving 550 rads of X-rays.

Parameters	Days after irradiation								
	0	7	10	12	14	16	19	21	24
ZPP µg/gm Hb	1.9 ± 0.1	2.1 ± .2	2.2 ± .3	2.2 ± .5	2.7 ± .5	3.2 ± .4	3.8 ± .4	3.7 ± .1	2.4 ± .1
Percent HcT	50 ± 2	38 ± 1	32 ± 1	25 ± 1	21 ± 1	17 ± 1	38 ± 1	38 ± 2	42 ± 1
Hb g/dl	15.4 ± .7	12.6 ± .2	10.9 ± .7	9.0 ± .3	7.6 ± .3	7.2 ± .4	10.4 ± .8	12.6 ± .5	12.7 ± .2
RBC x 10^6	8.66 ± .45	7.02 ± .18	6.35 ± .14	5.07 ± .14	4.33 ± .19	3.94 ± .19	4.70 ± .36	5.18 ± .20	7.44 ± .37
MCV μm^3	58 ± 1	54 ± 1	50 ± 1	50 ± 2	48 ± 2	44 ± 1	59 ± 4	73 ± 2	60 ± 2
MCH pg	17.7 ± .4	18.1 ± .9	17.3 ± .9	17.8 ± .5	17.7 ± .8	18.0 ± .4	22.5 ± 1.2	24.3 ± .6	17.2 ± .8
MCHC g/dl	30.6 ± .4	33.3 ± .9	34.0 ± 3.7	35.6 ± 1.4	36.6 ± 1.3	40.8 ± 1.1	40.1 ± 1.1	33.5 ± 1.7	29.7 ± .6

To determine if the elevated ZPP levels might simply be the result of increased reticulocyte numbers or a response to erythropoietin, 6 mice were treated with 5 units of erythropietic given i.p. These mice exhibited an increased reticulocyte count from 1% on day 0 to 7% to day 3 post treatment; however, no change in the ZPP level was observed.

Serum Iron, Total Iron Binding Capacity, Unbound Iron Binding Capacity, and Percent Iron Saturation Following Irradiation

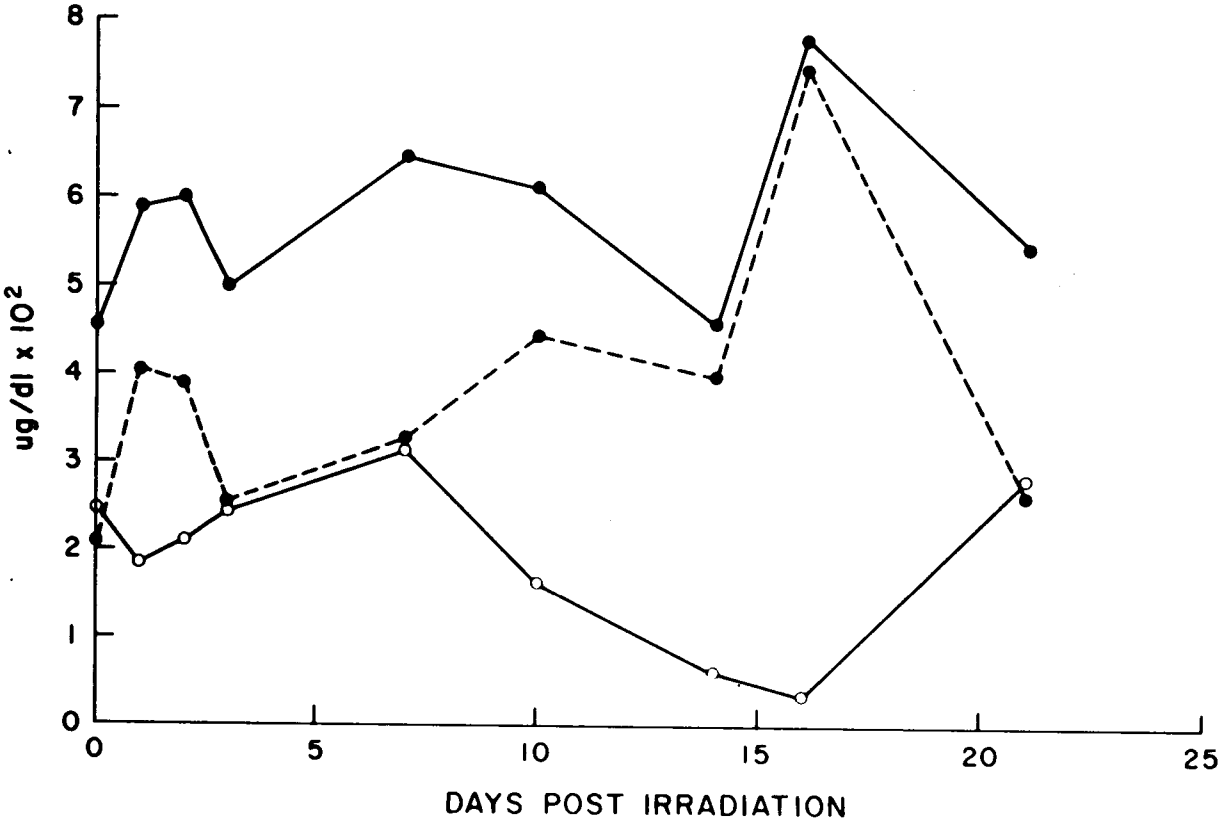
Elevation of ZPP is a diagnostic indicator of iron deficiency as well as of lead poisoning. Therefore, it was important to determine the status of iron metabolism in the irradiated mice. Radiation, by halting erythropoiesis, accelerating the loss of red-blood cells, damaging the intestines, inhibiting the synthesis of liver and serum proteins, and, later during the recovery phase, accelerating erythropoiesis, has the protential for perturbing iron metabolism (174,175). Thus it was necessary to assess whether iron deficiency was responsible for ZPP elevation following whole-body irradiation. The ZPP level and four diagnostic indicators of plasma iron levels were determined and compared in mice on the indicated days after 550 rad X-irradiation. The four diagnostic indicators of iron metabolism measured were the serum iron level, TIBC, UIBC, and percent iron saturation. The serum iron is the actual quantity of iron (mostly bound to transferrin) in serum. The TIBC reflects the levels of transferrin in the serum and is the total amount of iron that could be carried in the serum if all transferrin-iron binding sites were saturated. The UIBC is the amount of transferrin not

bound to iron and is the TIBC minus the serum iron level. These values are shown in Figure 5A. The percent iron saturation is the serum iron/TIBC x 100, shown in Figure 5B. This value drops below 16% in iron deficiency anemia (171). As shown in Figure 5B, the percent iron saturation never dropped below 50% during the course of these experiments.

The ZPP for these animals is shown in Figure 4 in which the ZPP and bilirubin levels are compared. The ZPP peaked on day 14, then dropped slightly but remained elevated during the next 7 days. The hematocrit in this experiment (not shown) dropped to its lowest point on day 14 and rose to control levels between days 16 to 21. Between days 14 to 16, the serum iron rose from 400 µg/dl to 745, and the TIBC jumped from 460 to 775 µg/dl. With the sharp increase in erythropoiesis after day 16, the serum iron levels, TIBC, and percent iron saturation dropped and the UIBC increased. During iron deficiency anemia, the serum iron level decreases, the TIBC increases, and the percent iron saturation decreases (176). The exact opposite occurs in iron poisoning, lead poisoning, hemolytic anemia, and sideroblastic anemias in which serum iron and percent iron saturation increase and the TIBC decreases (176,177). Shortly prior to and during the time that ZPP levels were rising, serum iron, TIBC, and percent iron saturation all increased, thereby ruling out the possibility that iron deficiency anemia induced by whole-body irradiation was the cause of ZPP elevation in irradiated mice.

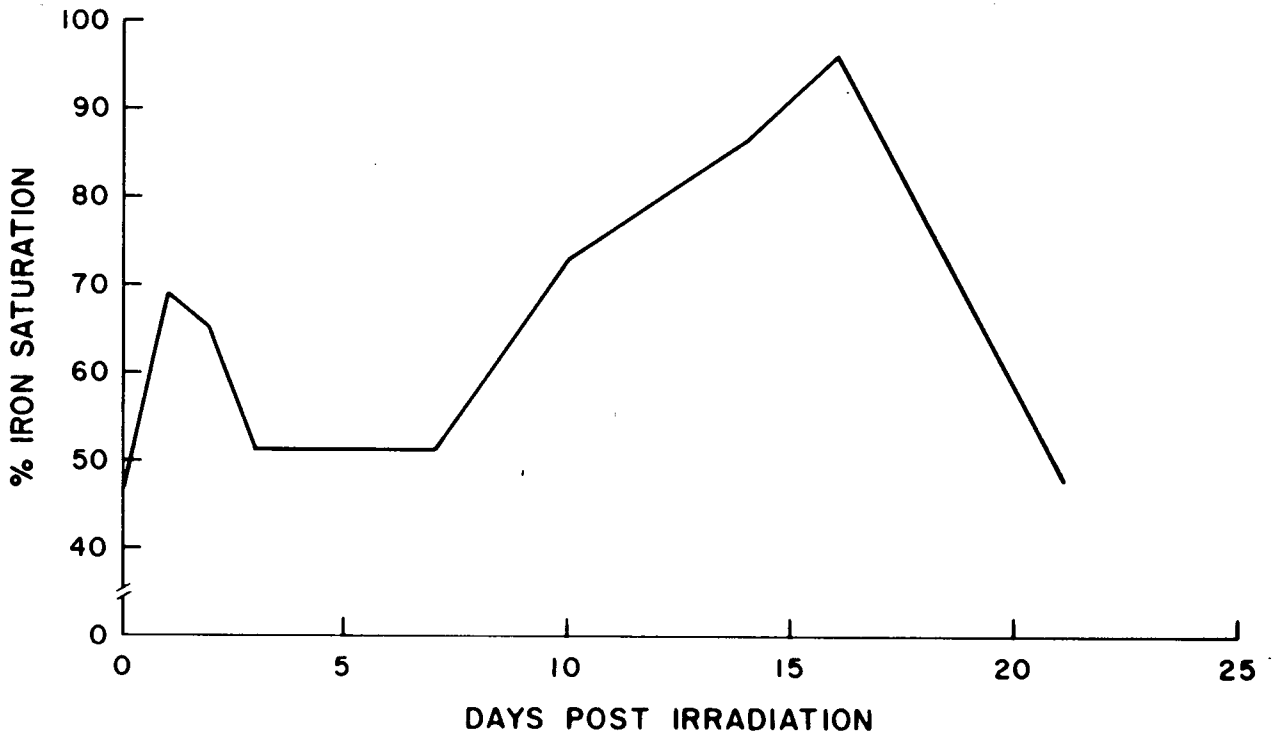
Effects of Radiation on Ferrochelatase Activity

ZPP becomes elevated following lead poisoning due to inhibiton of ferrochelatase (5,121). It was, therefore, necessary to determine



(A) Effect of X-irradiation on serum iron, total iron binding capacity, and unbound iron binding capacity. The mice used for these studies were the same as the ones described in Figure 4. (●---●) represents the serum iron, (●——●) represents the total iron binding capacity, and (o——o) represents the unbound iron binding capacity.

Figure 5. The effects of radiation on iron metabolism.



(B) The percent iron for each of the mice used for the experiment shown in Figure 4 was determined by dividing the serum iron level by the total iron binding capacity and multiplying by 100. The resutls are presented in the figure for the days post irradiation and are the averages from 5 mice for each point.

Figure 5 (continued)

whether ferrochelatase was inactivated to such a degree following whole whole-body irradiation that ZPP became elevated. Ferrochelatase activity was measured in spleen, liver, and red blood cells. The specific activities of ferrochelatase on days 9, 18, and 20 post irradiation are shown in Table 3. The data shown in Table 3 indicates that ferrochelatase activity was not reduced after irradiation but, in fact, was increased. Spleen ferrochelatase peaked on day 18 even though the spleen weight and the ZPP in circulating red cells were still increasing on day 20 (Figure 6). Liver ferrochelatase also rose during the post irradiation period. In red blood cells, ferrochelatase is found in the reticulocyte membrane (178) and is absent at the erythrocyte stage. The absence of activity in the blood on day 9 is due to the absence of reticulocytes. Ferrochelatase activity was elevated over control levels on days 18 and 20 post irradiation in all three organs tested. Therefore, the inhibition of ferrochelatase clearly cannot be an explanation for the radiation-induced elevation of ZPP.

Table 3. Ferrochelatase activity in irradiated mice.

Day	Ferrochelatase activity*		
	Spleen	Liver	Blood
Control	249 ± 66	1397 ± 439	13 ± 3
9	607 ± 14	3702 ± 700	0
18	1191 ± 38	2348 ± 179	83 ± 34
20	976 ± 180	2829 ± 40	124 ± 25

*pmol heme hr^{-1} mg^{-1} protein ± standard error, n = 2.

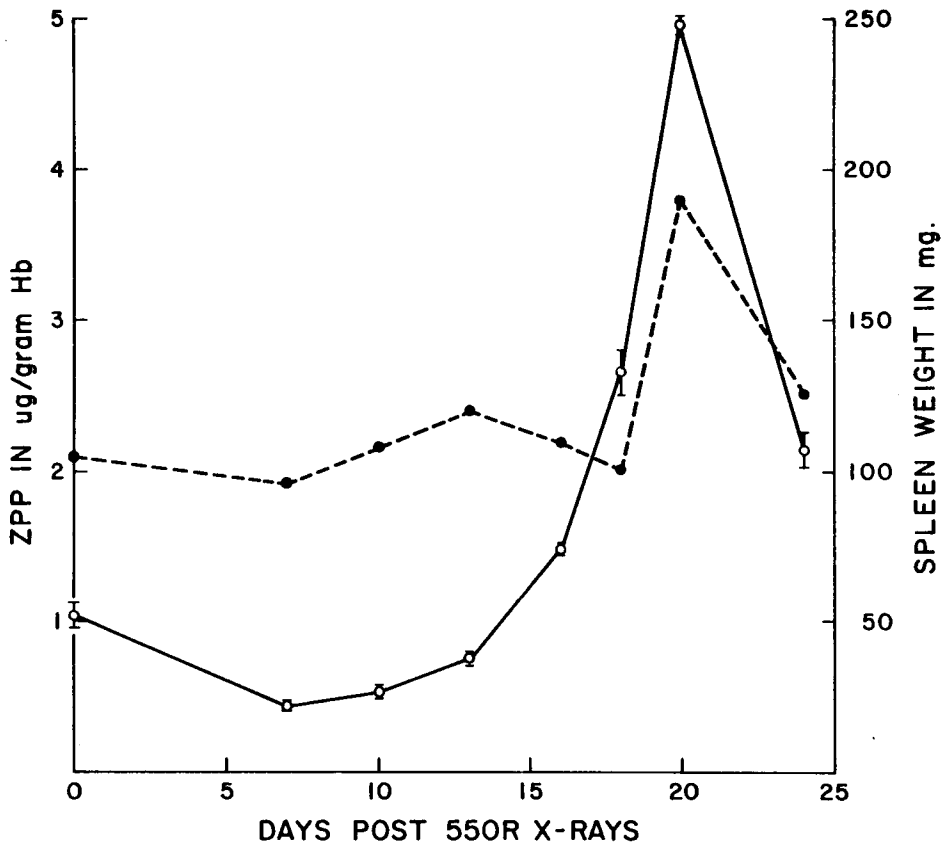


Figure 6. Peak weight in the regenerating spleen occurs at the same time as the peak elevation of ZPP.

C3H/HEJ male mice were irradiated with 550 rads of X-rays and then killed on the indicated days (5 mice for each point). Five unirradiated mice served as controls. The ZPP levels were determined and the spleens were excised and weighed. The spleen weights (o——o) and ZPP levels (●---●) are shown.

Effect of the Radioprotectant Compound WR-2721 on Radiation-Induced Elevation of ZPP

WR-2721, 438 mg/kg, was administered i.p. 15 minutes prior to 550 rads of X-rays. The results of WR-2721 treatment are summarized in Figure 6 and may be compared to the radiation response in the absence of WR-2721 shown in Figure 2, p. 56. In WR-2721 protected animals, ZPP did not differ appreciably from control values. The hematocrit in the WR-2721 treated animals dropped to its low value of 31 on day 12. In untreated animals, the hematocrit dropped to 16 on day 13 (Figure 2). The spleen weights of the WR-2721 protected mice are also shown in Figure 7 and may be compared to those of the unprotected irradiated animals in Figure 4, p. 60 In both the protected and the unprotected animals, the spleen weights reached their lowest points on day 7 post irradiation and then dropped to similar lows, 22 mg in the unprotected and 28 mg in the WR-2721 protected animal. Regeneration was faster in the protected mice than in the unprotected animals. Spleen weight peaked in the protected mice on day 16 to 105 mg, while in the unprotected animals, the peak spleen weight (250 mg) was on day 20.

Splenectomy Delays Radiation-Induced Elevation of ZPP

Mice were splenectomized and 1 week later received 550 rads of X-rays. The ZPP and hematocrits were measured on the day post irradiation indicated in Figure 8. The hematocrit in both the splenectomized and unsplenectomized irradiated mice decreased to similar low points, 13% and 16%, respectively. The recovery of

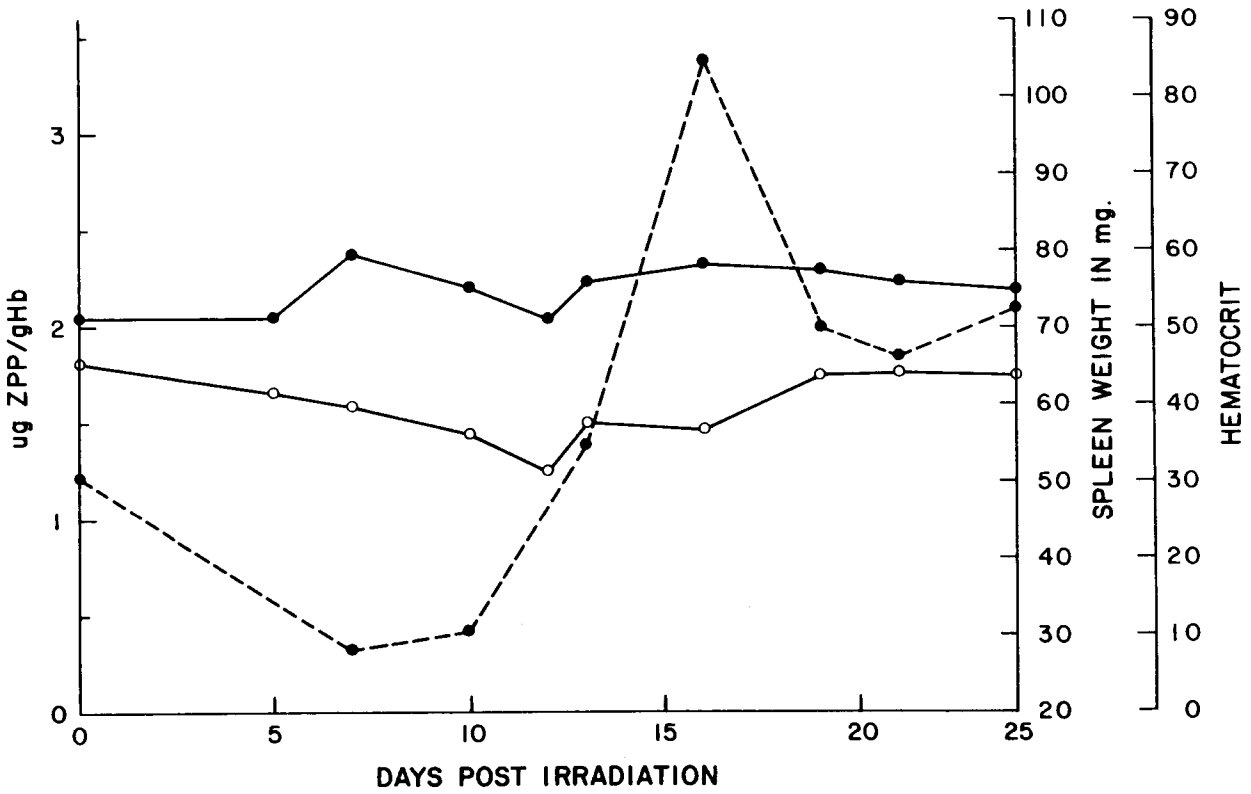


Figure 7. WR-2721, a radioprotectant, eliminates the radiation-induced elevation of ZPP.

WR-2721 was administered i.p. at a concentration of 438 mg/Kg body weight 15 minutes prior to administering 550 rads of X-rays. The average ZPP (●——●), hematocrit (o——o), and spleen weight (●--●) of 5 mice each are shown for the indicated days post irradiation.

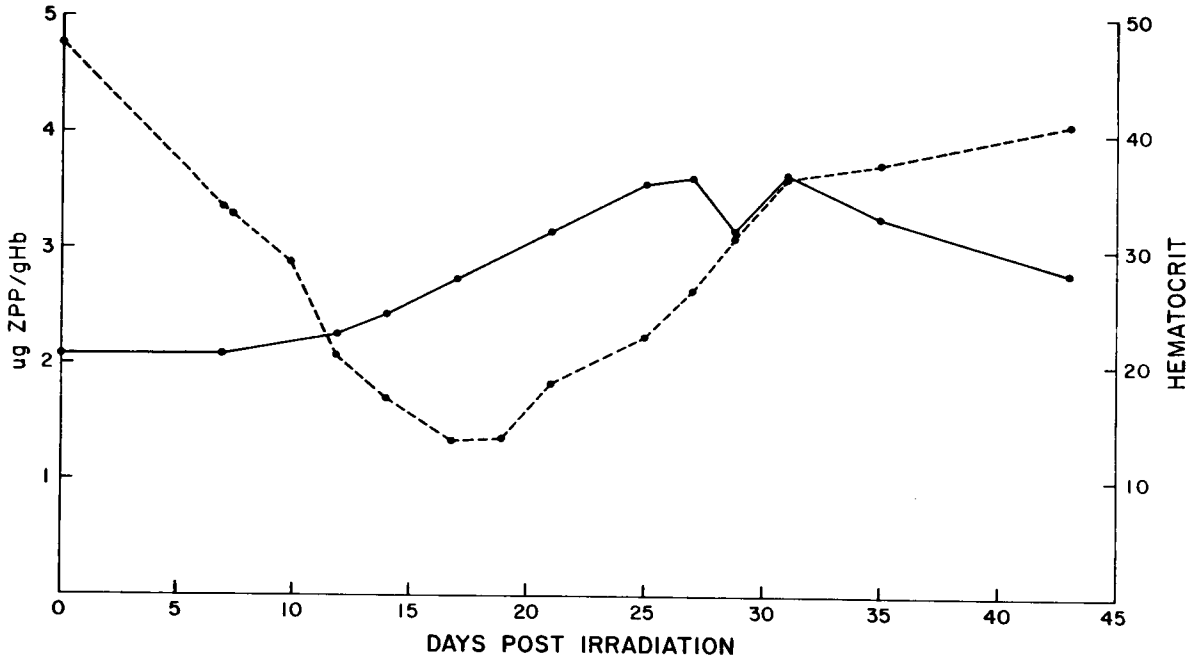


Figure 8. Splenectomy delays the radiation-induced elevation of ZPP.

The mice were splenectomized 1 week prior to receiving 550 rads of X-rays. Hematocrit (●--●) and ZPP (●—●) levels were determined at the indicated times post irradiation. Each data point is based on averages of 5 mice each.

erythropoiesis from this low point as indicated by the return of the hematocrit to normal levels was much slower in splenectomized mice (24 days) than for the unsplenectomized animals shown in Figure 2, p. 56 (10 days).

The rate of increase of ZPP is also much slower in the splenectomized mice, indicating a correlation between recovery of ZPP elevation with recovery of erythropoiesis. One other interesting observation to be noted in comparing Figure 2 and 7 is that the rate of decrease of ZPP after it reaches its peak value is similar to its rate of increase. In the experiment summarized in Figure 2, a rapid rise in ZPP between days 16-20, followed by an equally rapid decrease from days 20-24, was observed. This relationship between the rates is also evident with the splenectomized mice.

Combined Effect of Lead and X-irradiation

Mice were poisoned with lead and divided into two equal groups. Group I continued to receive lead acetate in the drinking water while Group II was removed from the lead regimen. One-half of the mice in each group was irradiated with 550 rads and the ZPP levels in all four groups were monitored. Figure 9A shows the results for unirradiated mice that continued to receive lead. The ZPP rose slowly over a 35-day period with the hematocrit remaining essentially unchanged. In the irradiated mice that continued to receive Pb, the hematocrit dropped, reached a minimum, and then began to rise (Figure 9B). The ZPP began to rise at this time as was previously seen in Figure 2, but the maximal ZPP reading was higher than was

Figure 9. (A-D) The combined effects of lead poisoning and X-irradiation on the elevation of ZPP.

C3H/HEJ male mice were lead poisoned by the addition of lead acetate (5×10^{-2} M) to their drinking water prior to 550 rads X-irradiation. At the time of irradiation, the average blood lead level was 2.8 parts per million (ppm). The average blood lead level of control mice not exposed to the lead-treated water was 0.16 ppm.

9A shows the results with unirradiated lead-poisoned mice that continued to receive Pb^{2+};

9B irradiated animals that continued to receive lead-containing water;

9C represents the unirradiated mice; and

9D irradiated mice placed on normal water.

The ZPP levels shown are based on averages of 4 or 5 animals per data point. The ZPP and hematocrit levels of all sets of animals were followed at various times post irradiation. Blood samples 30-40 µl were obtained from each animal every 6 days. By staggering several groupings, samples could be obtained every 2-3 days. The ZPP levels are represented by (●——●) and the hematocrits by (●--●). A solid line in Figure 8-C shows the ZPP level in non-lead poisoned unirradiated controls.

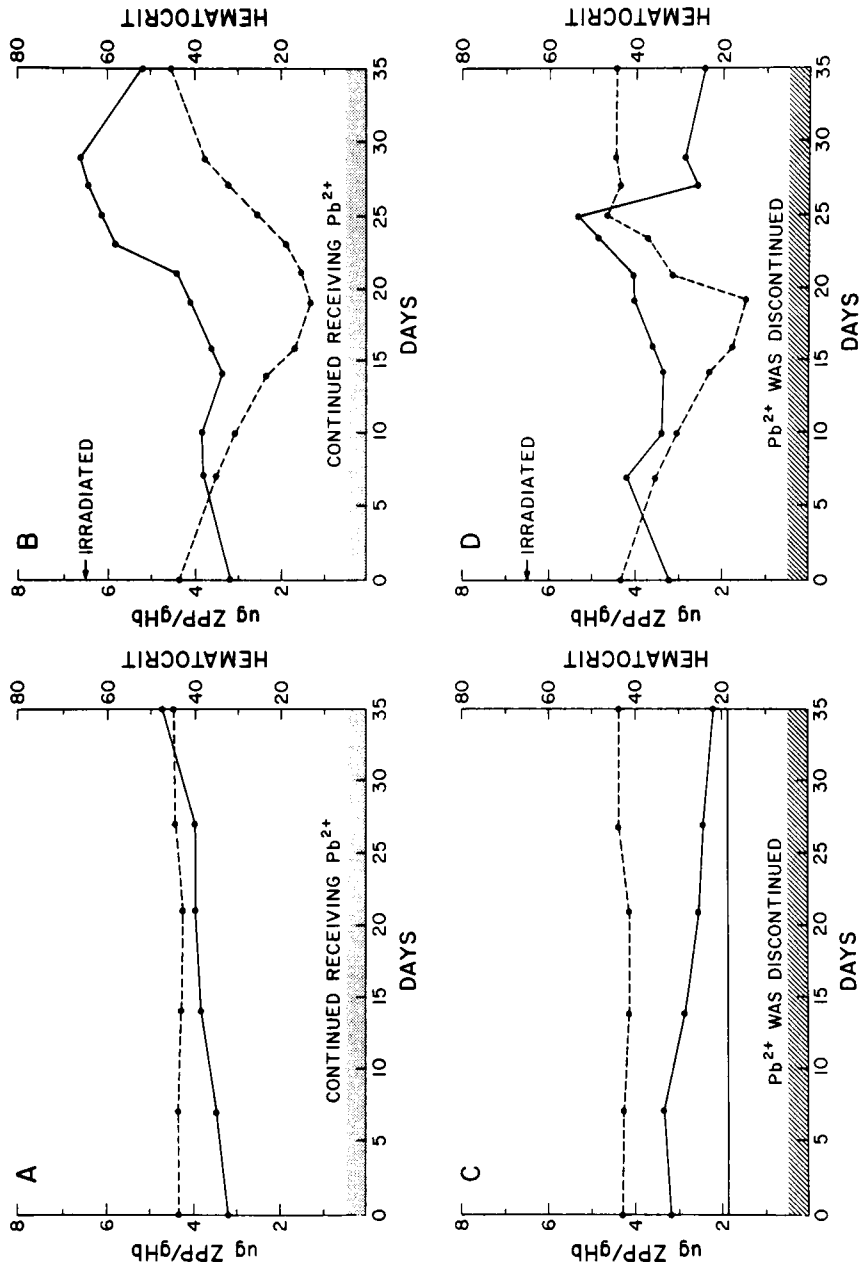

A
B
C
D
IRRADIATED
CONTINUED RECEIVING Pb^{2+}
Pb^{2+} WAS DISCONTINUED
ug ZPP/gHb
HEMATOCRIT
DAYS

Fig. 9

caused from radiation alone. Thus, the effect of irradiation and lead on ZPP elevation appears to be additive.

In the mice placed on a lead-free regimen and not irradiated, the ZPP dropped slowly during the course of the experiment from a slightly elevated value and returned to normal (Figure 9C). In the irradiated lead-poisoned mice that were put on a normal regimen (Figure 9D), the typical post-irradiation spike in ZPP was observed but it came a bit later (as did the recovery of erythropoiesis) than occurred in mice that had never been exposed to Pb (Figure 2, p. 56).

Discussion

Prior to this report, the only conditions reported to cause an elevation of ZPP were iron deficiency anemia and lead poisoning (5). We have demonstrated a third condition that results in an elevation of ZPP in mice and rats, namely whole-body X-irradiation. There was a threshold radiation dose below which ZPP elevation did not occur. The ZPP elevation varied both between strains of mice and between species. The lowest effective dose in C3H/HEJ mice was 450 rads and in both CD-1 mice and Sprague Dawley rats it was 600 rads.

There was a correlation between the recovery of the hematopoietic system and the elevation of ZPP. This was clearly indicated by experiments in which we caused the rise in the hematocrit after irradiation to be delayed by splenectomy, lead poisoning, or changing X-ray dose. In all cases, the elevation of ZPP was correlated with the rise in hematocrit. Further evidence that the

elevation of ZPP was tied to the recovery of the hematopoietic system from radiation damage was that at radiation doses that cause hematopoietic death, ZPP elevation was not observed.

The elevation of ZPP was not observed after injection of erythropoietin, indicating that the synthesis of ZPP may not occur inadvertently during elevated hematopoiesis but that it occurred only during recovery of the hematopoietic system from chemical, radiation, or nutritional insult. A major difference between the elevation of ZPP during recovery from radiation damage and lead poisoning was the appearance of a "spike" in the radiation response that has not been reported in lead toxicity studies. In splenectomized mice, the spike was not as acute, both on the upward and downward sides, indicating that the spleen may be involved either with the production of a cohort of cells with high levels of ZPP, with the removal of this cohort, or both. As shown in Table 2, p. 62, a 44% increase in the red cell count occurred between days 21 to 24 post irradiation. The sharp decrease in ZPP during this same time could be accounted for if the red cells produced during this time had less ZPP than cells present between days 16 to 20. The cells with reduced ZPP levels would dilute out the high ZPP-containing cells, resulting in an overall decrease in the "average" ZPP level.

Changes in bilirubin, hemoglobin, and hematocrit have been reported to cause false elevations of ZPP when measured by a dedicated hematofluorometer (151,152). But, as we report here, these changes were not responsible for the radiation-induced elevation of ZPP.

The influence of hemoglobin and hematocrit variations on ZPP measurements arise from the design of the hematofluorometer (151,152); these variations were eliminated as causes of error in our study by allowing sufficient time for the red blood cells to settle on the coverslip (152). Comparison of bilirubin and ZPP following irradiation shows that ZPP was still elevated at times when bilirubin has returned to normal levels. Furthermore, the fluorescence from the highest amount of bilirubin that we observed (1.06 mg/dl) would only increase the ZPP level by about 0.5 µg ZPP/g Hb. One other artifact considered was free protoporphyrin which has a fluorescence peak at 632 nm. Elevation of free protoporphyrin occurs in iron deficiency and lead poisoning (121). Rabbits receiving a single injection of lead acetate showed an initial increase in free protoporphyrin and ZPP (179). Elevation of free protoporphyrin also occurs with increased erythropoiesis in response to hypoxic conditions (180). The hematocrit in mice maintained under hypoxic conditions increased to 75 without any change in ZPP (Walden, T.L, and Farkas, W. R., unpublished results). Prototype hematofluorometers for ZPP detection described earlier in the literature had wider fluorescence detection windows and picked up free protoporphyrin as well as ZPP (6,8). The hematofluorometer used in these studies was specifically dedicated to the quantiation of ZPP and does not register free protoporphyrin.

Measurements of the serum iron, total iron binding capacity, and the percent iron saturation following irradiation indicated that iron deficiency was not the explanation of radiation-induced ZPP

elevation. ZPP becomes elevated during lead poisoning due to inhibition of ferrochelatase (5,121), the enzyme that inserts iron into the protoporphyrin ring. Presumably, ZPP is formed by a nonenzymatic reaction between zinc and protoporphyrin (5). Radiation, however, did not diminish ferrochelatase. The ZPP elevation resulting from irradiation and lead poisoning was additive. A dose of 900 rads given to rats has been reported to cause an uptake of zinc by spleen mitochrondria (181). That observation may be significant since ferrochelatase is a mitochondrial enzyme (123). Attempts to increase ZPP levels in normal mice by daily injections of zinc and/or by placing zinc in the drinking water did not succeed (data not shown).

The biological significance of elevated levels of ZPP are, for the most part, unknown. It is known that the ZPP complex is unable to transport oxygen since it is diamagnetic (144). During the period of rapid recovery of erythropoiesis from radiation damage, the formation of ZPP may be preferable to the accumulation of free protoporphyrin. Hemin and ZPP are able to stimulate and maintain protein synthesis in both intact reticulocytes and cell free hemolysates (161,162). Hemin and other metalloporphyrins (ZPP has not been tested) can bind to the hemin controlled translational repressor (HCR), inhibiting it and preventing the phosphorylation and inactivation of elongation factor 2 (161). Protoporphyrin can also bind to and inactivate the HCR, but unlike hemin and ZPP, it cannot stimulate or maintain protein synthesis (161).

ZPP effects the activity of at least one enzyme, heme oxygenase. Injection of ZPP at a dose of 50 μmole/kg body weight caused a 50% decrease in heme oxygenase activity 16 hours after injection in rats (118). Heme oxygenase catalyzes the breakdown of heme to bilirubin. ZPP is not degraded by the enzyme and acts as a competitive inhibitor (118). ZPP also effects other enzyme systems since it caused an 18% increase in oxygen consumption in a rat liver homogenate while protoporphyrin had no effect (160). Hemin, as an inhibitor, and protoporphyrin, as an activator, have been proposed as a regulatory system for guanylate cyclase (182). Possibly ZPP has some as yet undiscovered role in regulating the hematopoietic system during recovery from chemical, radiological, or nutritional insults. These studies indicate that damage to the hematopoietic system and subsequent recovery are responsible for ZPP elevation. If true, then bone marrow suppressive drugs should elicit a similar effect. Studies showing elevation of ZPP by hematotoxic drugs are now in progress.

The magnitude of the elevation of ZPP due to lead poisoning is very species dependent. In humans, ZPP elevation during lead poisoning is much more extensive than in mice and rats (179). If this is also true of the ZPP elevation due to radiation, it may be a useful indicator of the status of the hematopoietic system in patients being treated with radiation and hematosuppressive drugs. Finally, it is unlikely that a blood sample with an elevated ZPP discovered during a screen for lead poisoning would have come from a

subject who had received high doses of radiation. Nevertheless, it should be kept in mind that factors other than lead poisoning and iron deficiency can cause elevated ZPP.

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