LONG-TERM ADMINISTRATION OF MASSIVE DOSES OF Sn-PROTOPORPHYRIN IN ANEMIC MUTANT MICE (sph^{ha}/sph^{ha})

BY SHIGERU SASSA,* GEORGE S. DRUMMOND,* SELDON E. BERNSTEIN,‡ AND ATTALLAH KAPPAS*

*From *The Rockefeller University Hospital, New York 10021; and ‡The Jackson Laboratory, Bar Harbor, Maine 04609*

The present study was undertaken to examine the long-term hematological and histopathological effects of the administration of very large doses of the heme oxygenase inhibitor, Sn-protoporphyrin, in genetically mutant mice with hemolytic anemia (sph^{ha}/sph^{ha}). The study was prompted by the potential for clinical use of this enzyme inhibitor to suppress severe neonatal hyperbilirubinemia in humans. This condition is currently treated principally by phototherapy and by exchange transfusions (1) but these treatments are not without drawbacks (1). Moreover, these modalities of therapy are directed towards the complex problem of disposing of bilirubin after, rather than before, it has been formed in the heme oxidation system.

In contrast, in previous studies from this laboratory (2, 3), we have proposed the idea that it might be possible, by using the heme oxygenase inhibitor Sn-protoporphyrin, to inhibit the formation of bilirubin, thus approaching severe neonatal hyperbilirubinemia in a chemopreventive manner. Among a series of related compounds that we examined, Sn-protoporphyrin was not only the most potent inhibitor in vitro of the activity of microsomal as well as homogenously purified heme oxygenase (2–4), but could also suppress hyperbilirubinemia in the rat neonate (2, 3) and in various forms of naturally occurring or experimentally induced jaundice in animals and man (5, 6).

Since Sn-protoporphyrin has not, in our studies, been associated with any significant toxicity after its acute administration in animals or man, we felt it important to investigate the long-term effects of large amounts of this metalloporphyrin in genetically anemic mutant mice with hemolytic anemia (sph^{ha}/sph^{ha}), a disease in which very severe hemolysis and excessive hyperbilirubinemia are conspicuous features. The results of this study confirm no apparent toxic effects of Sn-protoporphyrin over 8 mo, in a marginally viable strain of mutant mice with profound hemolytic anemia.

Materials and Methods

Mice. The mutant mice used in this study were animals with hemolytic anemia ($WBB6F_1$-sph^{ha}/sph^{ha}) produced by mating heterozygotes from two different inbred

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strains, WB/Re and C57BL/6J, reared at The Jackson Laboratory. The origin of the mutant and its pathophysiological characteristics have been described previously (7). Animals were housed under conditions approved by the American Association of Laboratory Animals Center and fed Purina Lab Chow and water ad lib. Animals used were of both sexes and were aged 8–10 mo, except in the 32-wk study in which 4-mo-old animals were used. Because of the extreme rarity of this strain of mice, experimental groups could not be large (two animals per data point; seven animals in each group in the 32-wk study) and statistical analysis of data was not performed unless it was applicable.

Sample Preparation. Blood was obtained from the retroorbital sinus for the determination of plasma bilirubin, Sn-protoporphyrin, and free erythrocyte protoporphyrin, as well as activities of δ-aminolevulinate (ALA)[1] dehydratase and porphobilinogen (PBG) deaminase. Mice were killed by cervical dislocation. Liver, kidney, and spleen were removed for the isolation of microsomes and the determination of cytochrome P-450 and heme oxygenase activity.

For histochemical studies, tissues were fixed in Tellyesniczky's fixative (formalin/acetic acid/alcohol) for 24 h before paraffin imbedding and sectioning at 7 μm. Sections were stained with hematoxylin and eosin.

Assays. Erythrocyte protoporphyrin, ALA dehydratase activity, and PBG deaminase activity were determined as described previously (8). ALA synthase activity and microsomal heme oxygenase activity was assayed as described previously (9). Cytochrome P-450 was determined according to the method of Omura and Sato (10). Sn-protoporphyrin concentration was determined by a fluorometric method developed in our laboratory (11). Plasma bilirubin concentrations were quantified after the method of Roth (12). Fluorometric determinations (for free erythrocyte protoporphyrin, Sn-protoporphyrin, plasma bilirubin, and PBG deaminase activity) were made using a Hitachi/Perkin-Elmer MPF 4 fluorescence spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) equipped with a R928 photomultiplier (9). The instrument was calibrated daily with 0.1 μM coproporphyrin III in 0.14 N HCl.

Sn-protoporphyrin. Sn-protoporphyrin (Porphyrin Products, Logan, UT) was dissolved in 0.5 N NaOH and diluted with 0.9% NaCl as described previously (14). The pH of the solution was adjusted to ~8 with 0.5 N HCl before subcutaneous injection. Control animals received an equivalent volume of vehicle prepared without Sn-protoporphyrin.

Results

Accumulation of Sn-protoporphyrin in the Liver and Kidney. Sn-protoporphyrin concentrations were determined fluorometrically in homogenates from the liver and kidney in sph^{ha}/sph^{ha} mice 2 wk after the first injection. Sn-protoporphyrin content in both organs increased proportionately with increasing doses of the compound (Fig. 1). Repeated administration of 100 μmol/kg body weight caused marked tissue accumulation. The spleen accumulated lower concentrations than the other two organs, e.g., one-fifth of the concentration in the liver (data not shown).

Effects of Sn-protoporphyrin on Heme Oxygenase Activity. The levels of heme oxygenase activity in sph^{ha}/sph^{ha} mice were examined after treatment with Sn-protoporphyrin. Animals were injected subcutaneously on day 0 with saline and either 10 μmol/kg or 100 μmol/kg Sn-protoporphyrin or five consecutive daily doses, subcutaneously, of 100 μmol/kg body weight of Sn-protoporphyrin. They were then sacrificed on day 14. As shown in Fig. 2, heme oxygenase activity in the liver, spleen, and kidney was decreased in a dose-dependent manner. Five

[1] *Abbreviations used in this paper:* ALA, δ-aminolevulinate; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PBG, porphobilinogen; RBC, red blood cell.

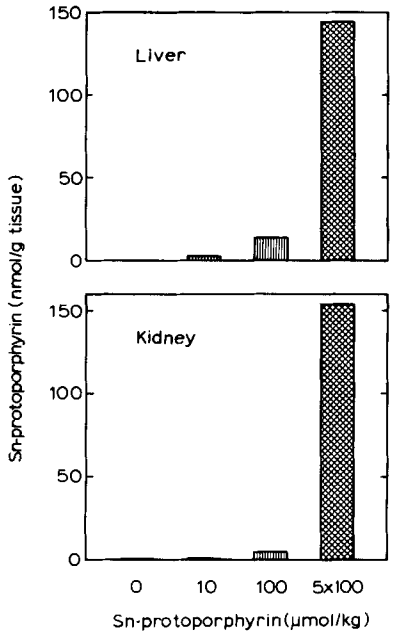

FIGURE 1. Tissue concentration of Sn-protoporphyrin. 8-mo-old female animals were used. Animals were treated with saline plus 10 or 100 μmol/kg Sn-protoporphyrin on day 0 or five consecutive daily doses of 100 μmol/kg Sn-protoporphyrin and sacrificed on day 14. Tissue concentration of the metalloporphyrin was determined fluorometrically as described in the text. Data are the mean of duplicate determinations for two animals for each point. Variations between two animals were within 15% of the mean.

injections each of 100 μmol/kg Sn-protoporphyrin resulted in ~80, ~70, and ~90% suppression of heme oxygenase activity in the liver, spleen, and kidney, respectively.

Effect of Sn-protoporphyrin on Plasma Bilirubin Concentrations. Fig. 3 shows the effect of Sn-protoporphyrin (100 μmol/kg body weight, s.c., once weekly) on bilirubin and Sn-protoporphyrin concentrations in plasma in sph^{ha}/sph^{ha} mice for a total of 16 wk. Plasma Sn-protoporphyrin concentration increased progressively during the treatment. In contrast, bilirubin concentration was decreased to ~50% 2 wk after treatment and remained at approximately that level for the full period of study.

Dose-Response Effects of Sn-protoporphyrin on Plasma Bilirubin Levels. In the course of this study, we observed that average plasma bilirubin levels in sph^{ha}/sph^{ha} mice varied from 2.0 to 3.7 mg/dl depending on their age, sex, housing conditions, and perhaps other unknown factors. However, the coefficient of variation in bilirubin concentration within the same sex from the same littermates was always very small (within 5% of the mean); thus, all experiments were performed using animals of the same sex from the same litter housed under identical conditions. Table I shows the effect of variable doses of Sn-protoporphyrin on plasma bilirubin levels 3 d after injection. Plasma bilirubin decreased proportionately with the increase in the dose of Sn-protoporphyrin.

Effects of Sn-protoporphyrin on Erythrocyte Heme Pathway Enzymes and Protopor-

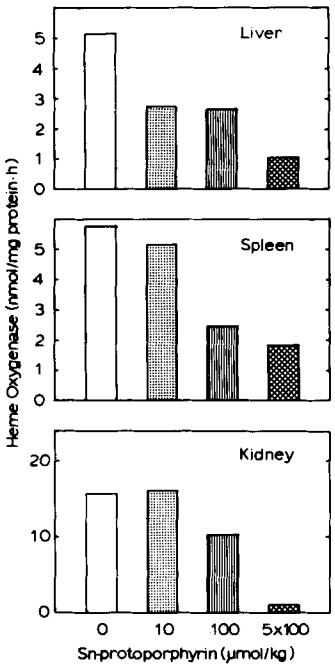

FIGURE 2. Effect of Sn-protoporphyrin on microsomal heme oxygenase activity. 8-mo-old female mice (sph^{ha}/sph^{ha}) were used. Animals were treated as described in the legend to Fig. 1. Heme oxygenase activity was determined using duplicate microsomal samples prepared from two livers or two spleens and four kidneys for each point, as described in the text.

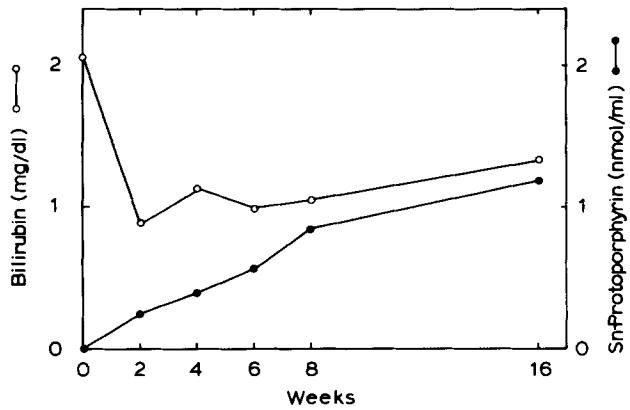

FIGURE 3. Effect of Sn-protoporphyrin on plasma bilirubin concentrations. 8-mo-old female animals were used. Animals were treated with Sn-protoporphyrin (100 μg/kg body weight, s.c.) once weekly during the experimental period. Blood specimens were collected from the orbital sinus without sacrificing the animals. Bilirubin and Sn-protoporphyrin concentrations were determined fluorometrically as described in Materials and Methods. It has been shown previously (2, 11) that the two fluorometric assays do not interfere with each other. Each point represents the mean of two mice. Variations between two animals were within 15% of the mean.

TABLE I

Dose-Response Effect of Sn-protoporphyrin on Plasma Bilirubin Concentration

Sn-protoporphyrin	No. of injections	Plasma bilirubin
μmol/kg		*mg/dl*
0	—	3.61 ± 0.15
10	1	3.14 ± 0.67 NS
100	1	2.43 ± 0.37 ($P < 0.01$)
100	3	1.87 ± 0.21 ($P < 0.001$)

10-mo-old male sph^{ha}/sph^{ha} mice were used for study. Sn-protoporphyrin was injected subcutaneously on day 0. The last group received Sn-protoporphyrin injections once daily on days 0, 1, and 2. Plasma bilirubin was determined on day 3 (72 h after the first injection) as described in Materials and Methods. Data are the mean of four determinations using two mice. NS, not significant.

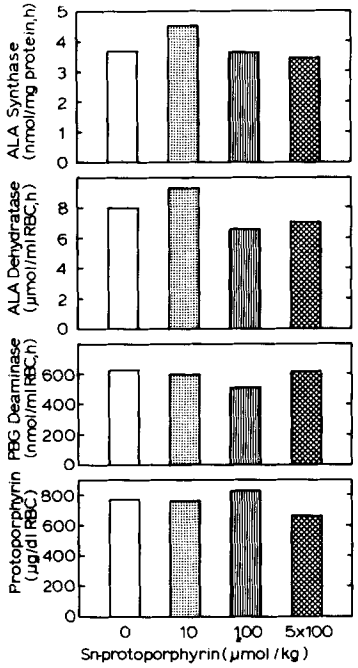

FIGURE 4. Effects of Sn-protoporphyrin on the activity of ALA synthase, ALA dehydratase, and PBG deaminase in erythrocytes. 8-mo-old female animals were used. Animals were treated as described in the legend to Fig. 1. Enzyme assays were carried out using whole blood, as described in Materials and Methods. Each point represents the mean of duplicate assays. Variations between the two animals were within 10% of the mean.

phyrin Concentrations. Effects of Sn-protoporphyrin on the activities of heme pathway enzymes and protoporphyrin concentrations in erythrocytes were also examined. As shown in Fig. 4, Sn-protoporphyrin treatment did not significantly alter the activities of ALA synthase, ALA dehydratase, and PBG deaminase, or the concentration of erythrocyte protoporphyrin.

Effects of Sn-protoporphyrin on Heme Pathway Enzymes and Cytochrome P-450 in the Liver and the Kidney. Fig. 5*A* shows the effect of Sn-protoporphyrin on the activities of ALA synthase, ALA dehydratase, and PBG deaminase in the liver of sph^{ha}/sph^{ha} mice. Sn-protoporphyrin treatment slightly decreased ALA dehydratase activity in the liver in a dose-dependent manner, while PBG deaminase activity was decreased slightly only at the two higher doses. Hepatic ALA synthase was suppressed ~40% by all three doses. The observed decreases in heme pathway enzymes did not appear to have significant consequences for heme formation in the liver and kidney since cytochrome P-450 content in both tissues remained unaffected by Sn-protoporphyrin treatment (Fig. 5*B*).

Sn-protoporphyrin Disappearance from Plasma. Sn-protoporphyrin was fluorometrically detectable in plasma within 1 h of its injection. It had a characteristic fluorescence emission spectrum (max_1 = 581 nm; max_2 = 635 nm) in a mixture of *N*-perchloric acid/methanol (1:1 vol/vol), thus permitting its sensitive detection in the presence of other porphyrins (11). Sn-protoporphyrin's disappearance from plasma involved complex kinetics, with an initial half-life of 4 h, followed by increased half-lives of 12 h and 3 d as the plasma level declined to very low levels (Fig. 6).

Effect of Sn-protoporphyrin on Hematological Indices. The effects on hematological indices of Sn-protoporphyrin- and vehicle-treated sph^{ha}/sph^{ha} mice were

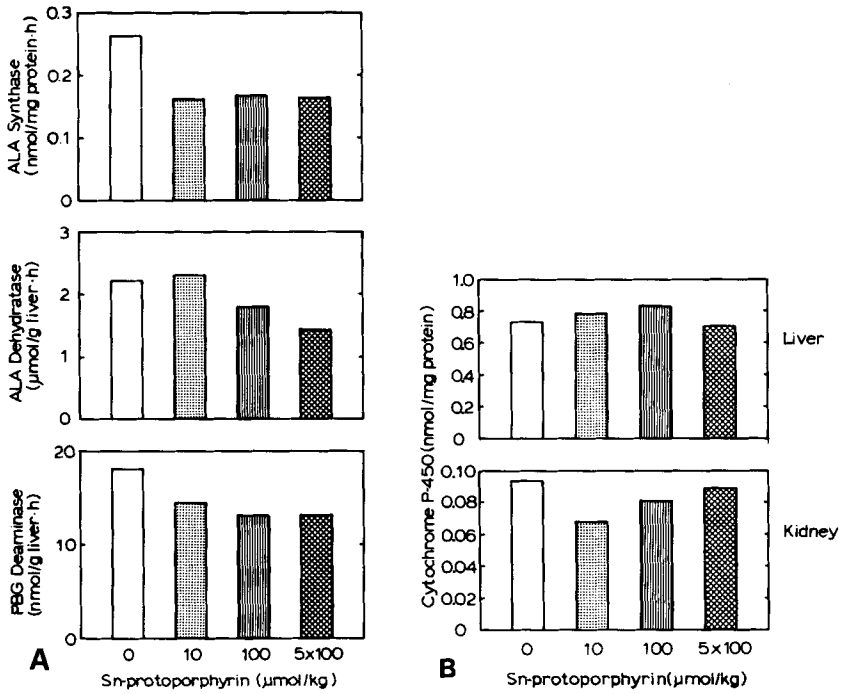

FIGURE 5. Effects of Sn-protoporphyrin on heme pathway enzymes and cytochrome P-450 content. (*A*) ALA synthase, ALA dehydratase, and PBG deaminase in the liver. (*B*) Cytochrome P-450 in the liver and the kidney. Animals were treated as described in the legend to Fig. 1. Enzyme assays and cytochrome P-450 determinations were carried out as described in Materials and Methods. Data are the mean of two mice.

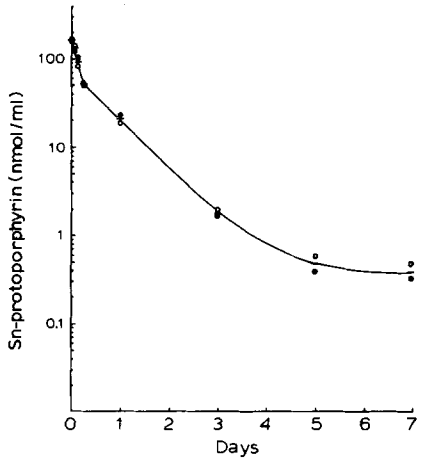

FIGURE 6. Sn-protoporphyrin disappearance from plasma. Plasma Sn-protoporphyrin concentration was determined fluorometrically (11) after a single injection of 100 μmol/kg body weight, s.c. Data are the mean of duplicate determinations for each of two 8-mo-old female mice.

TABLE II

Effects of Sn-protoporphyrin on Hematological Indices in sph^{ha}/sph^{ha} Mice

Hematological index	Vehicle			Sn-protoporphyrin		
	Pretreatment	12 wk	Posttreatment	Pretreatment	12 wk	Posttreatment
RBC ($\times 10^{12}$/liter)	4.80 ± 0.46	4.29 ± 0.09	4.40 ± 0.34	4.85 ± 0.08	4.62 ± 0.14	4.70 ± 0.23
Hematocrit (%)	27.3 ± 1.75	25.9 ± 0.53	24.5 ± 1.58	27.6 ± 0.39	26.1 ± 0.63	26.4 ± 2.69
MCV (μ^3)	57.0 ± 2.60	60.23 ± 0.67	55.9 ± 5.7	57.0 ± 0.68	56.6 ± 1.33	56.2 ± 4.64
Hemoglobin (g/dl)	6.36 ± 0.54	6.00 ± 0.12	5.70 ± 0.40	6.53 ± 0.17	6.01 ± 0.14	5.70 ± 0.48
MCHC (g/dl)	23.3 ± 1.68	23.21 ± 0.25	23.3 ± 1.58	23.7 ± 0.46	23.0 ± 0.36	21.6 ± 0.63
Protoporphyrin (μg/dl RBC)	980 ± 140	971 ± 39	1,015 ± 46	1,023 ± 38	1,042 ± 29	960 ± 101
Reticulocytes (%)	94.5 ± 0.9	96.3 ± 0.2	97.4 ± 1.8	93.8 ± 1.3	96.8 ± 0.4	98.2 ± 1.0

Seven mice (4 mo old) in each group were treated with vehicle or Sn-protoporphyrin (100 μmol/kg, s.c., once weekly) for up to 32 wk. Data shown for posttreatment are hematological indices (mean ± SE) 1 wk after the last injection. Weekly hematological examinations during the treatment period did not show significant changes between the vehicle-treated control and Sn-protoporphyrin-treated animals.

determined at regular intervals during the 32 wk of treatment (100 μmol/kg body weight, s.c., once weekly). Each group consisted of seven female mice. Results are shown for the pretreatment, treatment, and the posttreatment periods (Table II). There were small, age-dependent changes in most of the hematological indices in both vehicle-treated control and Sn-protoporphyrin-treated animals. Both groups of animals showed similar hematological values characteristic of mice with this severe chronic form of hemolytic disease. Sn-protoporphyrin treatment had no significant effects on hematological indices (Table II).

Organ Weights and Histopathology of sph^{ha}/sph^{ha} Mice Treated with Sn-protoporphyrin. Table III shows the effects on organ weights, gall stones, and histopathology of vehicle-treated controls and mice treated with Sn-protoporphyrin (100 μmol/kg body weight, s.c., once weekly) for 32 wk. There were no

TABLE III
Long-term Effects of Sn-protoporphyrin on Organ Weights and Histopathology

	Organ weight after treatment with:			
	Vehicle		Sn-protoporphyrin	
	Mean ± SE	Percent of body weight	Mean ± SE	Percent of body weight
	g	%	*g*	%
Body wt.	28.05 ± 0.61	100	28.84 ± 0.76	100
Liver	2.24 ± 0.09	7.99	2.68 ± 0.12	9.29 ($P < 0.02$)
Spleen	1.39 ± 0.02	4.96	1.34 ± 0.11	4.64 NS
Kidney	0.36 ± 0.01	1.28	0.44 ± 0.02	1.53 ($P < 0.02$)
Femur	0.09 ± 0.002	0.32	0.09 ± 0.003	0.31 NS
Gall stones (μg/mouse)	175		140	

Seven mice (4 mo old) in each group were treated with vehicle or Sn-protoporphyrin (100 μmol/kg, s.c., once weekly) for 32 wk. Animals were sacrificed 1 wk after the last injection and organ weights were examined. NS, not significant.

differences in body weights, spleen sizes, or femur sizes between control and experimental groups. By contrast, there were increases of a low order of magnitude in liver size and kidney size (expressed as the organ weight as percent of total body weight) after prolonged Sn-protoporphyrin treatment. Average weights of gall stones were smaller (~20%) in Sn-protoporphyrin-treated animals than in controls; however, no statistical comparison could be made since gallstones had to be pooled for each group for determination of their dry weights. Representative figures for gall bladders containing stones are shown in Fig. 7 for each group.

Histopathological examinations revealed many well-demarcated orange-brown pigment concretions within cell clusters in the liver in both control and Sn-protoporphyrin-treated groups (Fig. 8*A*). Many highly active hematopoietic foci were present in the liver in both groups of animals. Spleens from both groups showed many scattered pigment granules and large black concretions (Fig. 8*B*). Kidneys also contained massive orange inclusions in the tubules but none in the medulla or in the glomerulae (Fig. 8*C*). Bone marrow and skin samples appeared generally normal in both groups of animals (Fig. 8*D*). The massive pigment granules in the liver, spleen, and kidney were typical of those found in severely hemolytic animals and there were no significant histopathological changes specifically attributable to treatment with Sn-protoporphyrin.

Discussion

The hemolytic anemic mutant mouse (sph^{ha}/sph^{ha}) represents an extremely useful model system for the study of hyperbilirubinemia since, in this mutant, red cell survival time is <1.5 d, compared with ~60 d in normal control animals (8). As a consequence, affected homozygotes have markedly elevated serum bilirubin levels (13, 14). These animals also have markedly decreased hemoglobin, hematocrit, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) and greatly elevated reticulocyte levels in

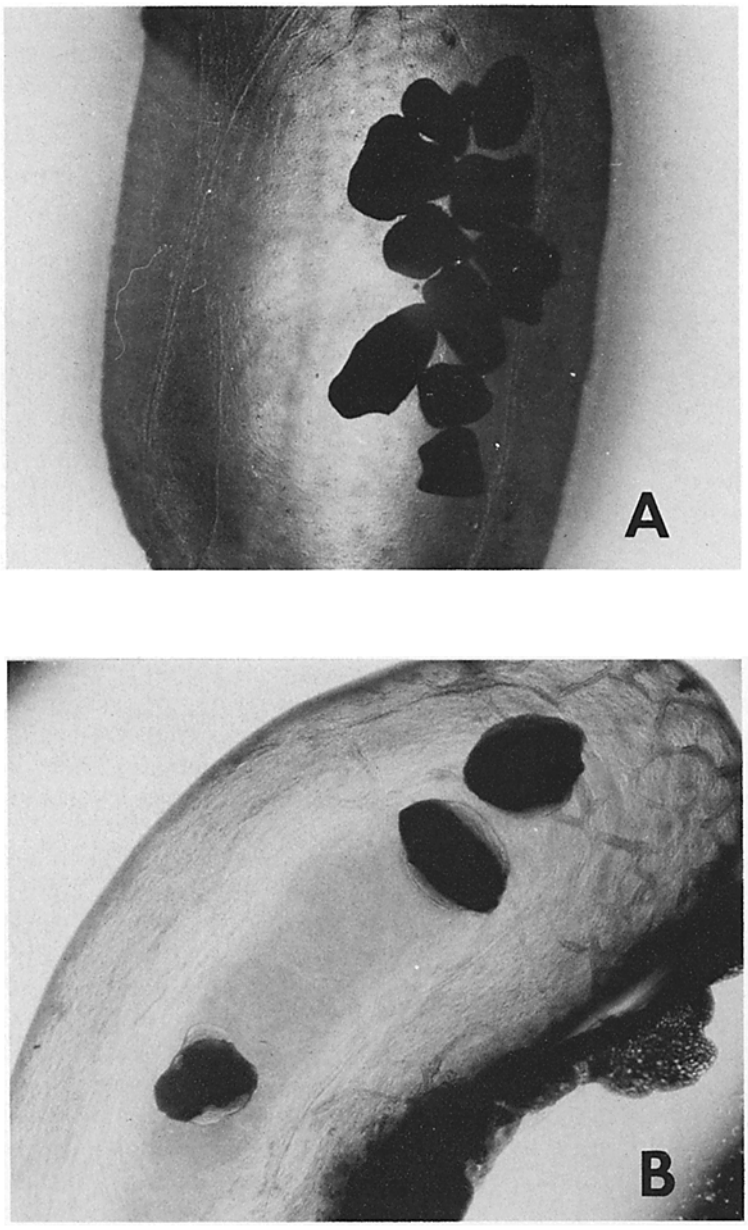

FIGURE 7. Gall bladders from sph^{ha}/sph^{ha} mice. (*A*) Gall bladder from a mouse (12-mo-old female) treated with vehicle for 32 wk. (*B*) Gall bladder from a mouse treated with Sn-protoporphyrin (100 μmol/kg body weight, once weekly) for 32 wk.

their circulation (~90% of red cells are reticulocytes). They also display an increased incidence of calcium bilirubinate gallstones as they age (15).

Our data on plasma bilirubin levels over 16 wk demonstrated that excessive hyperbilirubinemia in the affected homozygotes can be substantially decreased by Sn-protoporphyrin treatment. Sn-protoporphyrin also markedly suppressed

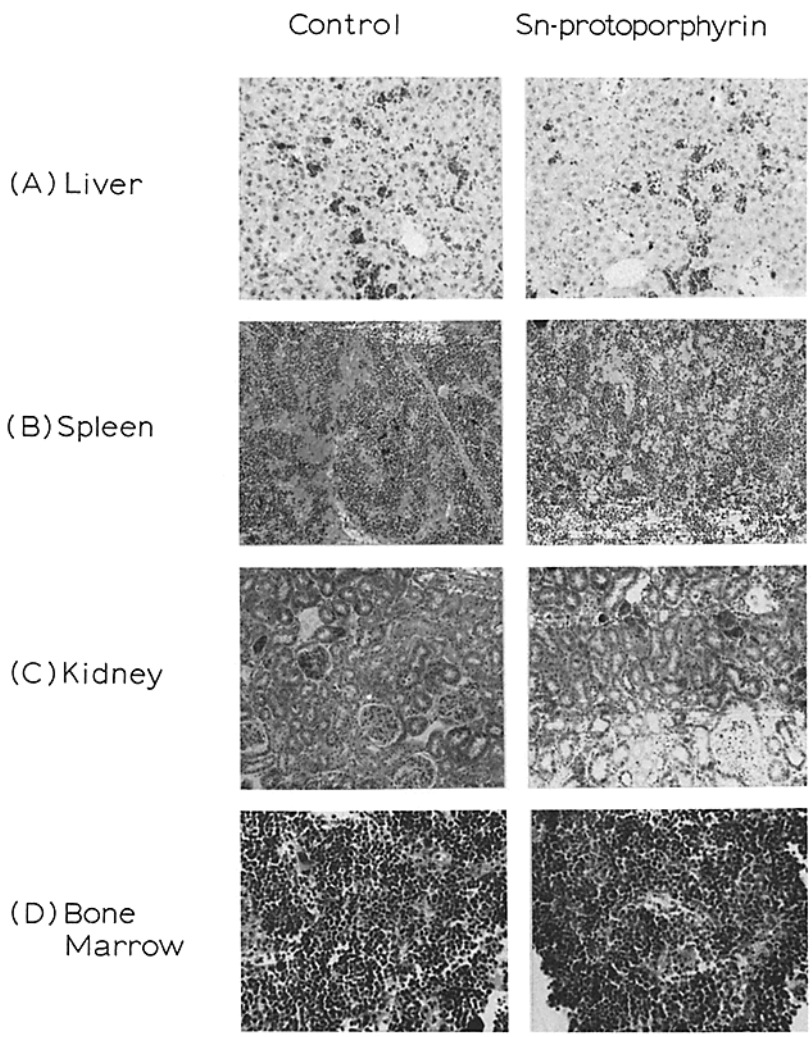

FIGURE 8. Histopathology of organs from sph^{ha}/sph^{ha} mice. Control: mice (12-mo-old female) treated with vehicle for 32 wk. Sn-protoporphyrin: mice treated with Sn-protoporphyrin (100 μmol/kg body weight) for 32 wk. Both groups showed orange concretions in the liver, kidney, and spleen that are due to their hemolytic disease. There are no obvious histopathological changes attributable to Sn-protoporphyrin treatment.

the activity of heme oxygenase in the liver, spleen, and kidney, while it did not significantly affect the enzymes in the heme biosynthetic pathway in the liver (Fig. 5), kidney (data not shown), spleen (data not shown), and erythrocytes (Fig. 4). Inhibition of heme oxygenase activity in the liver and the kidney did not have deleterious effects on the level of cytochrome P-450 in either organ (Fig. 5*B*). It is clear from these studies that continuous treatment with a large dose of Sn-protoporphyrin does not appreciably interfere with heme synthesis in erythroid cells, liver, and kidney, but does, in confirmation of earlier data (2, 3, 5, 6, 16),

significantly suppress heme oxygenase activity and plasma bilirubin concentration.

Sn-protoporphyrin has been shown to inhibit the activity of purified heme oxygenase (4) and heme oxygenase activity in microsomal preparations in vitro (2, 3). It also inhibits hepatic, splenic, and renal heme oxygenase activity in neonatal rats (2, 3), suppresses biliary bilirubin output derived from endogenous or exogenous heme sources (17), and substantially diminishes carbon monoxide production that results from the catabolism of endogenous and exogenous heme (18). These findings, together with those in the present study, confirm that this synthetic heme analogue inhibits heme oxidation in vivo (2, 3), even under markedly elevated conditions of bile pigment production.

When administered in vivo, Sn-protoporphyrin appears to be retained in tissues for considerable periods of time. The major portion of the plasma Sn-protoporphyrin was cleared with a half-life of 4 h (Fig. 6), while tissues such as liver, kidney (Fig. 1), and spleen accumulate the metalloporphyrin in a dose-dependent manner (19). Tissue accumulation of Sn-protoporphyrin for extended periods (detectable up to 2 wk after a single injection of 100 μmol/kg) can be explained in part by the fact that the metalloporphyrin cannot be oxidatively degraded by the microsomal heme oxygenase system (4). Long-term inhibition of heme oxygenase activity (up to 3 wk) after a single injection of Sn-protoporphyrin is also probably due to the metabolically stable nature of the metalloporphyrin. These data suggest that only a single injection may control hyperbilirubinemia in circumstances when excessive levels of this bile pigment may have a neurotoxic potential, as in a severely jaundiced premature infant.

Long-term treatment (32 wk) of 4-mo-old sph^{ha}/sph^{ha} mice with a very large dose of Sn-protoporphyrin (100 μmol/kg body weight), once weekly (total of 3,200 μmol) revealed no toxicity on erythroid, hepatic, and renal heme pathway enzymes. Hematological indices such as erythrocyte count, hemoglobin concentration, mean corpuscular volume (MCV), MCH, MCHC, reticulocyte count, granulocyte count, and platelet count were identical for vehicle-treated control and Sn-protoporphyrin-treated animals. Histopathological examinations of these tissues also demonstrated no pathological changes attributable to Sn-protoporphyrin. Treatment of mutant mice suffering a still more severe form of inherited hemolytic anemia, *ja/ja*, all of whom die within 10 d of birth, also did not reveal any sign of increased toxicity (data not shown).

In mice treated with Sn-protoporphyrin, the "free" heme concentration in liver cells may be increased as a consequence of the inhibition of heme oxygenase. A recent study from our laboratory (20) has, in fact, shown that an increase in free heme concentration occurs in the liver after Sn-protoporphyrin treatment, since the extent of heme saturation of hepatic tryptophan pyrrolase increases rapidly and markedly after Sn-protoporphyrin treatment. Excessive heme accumulation, however, does not occur, since it has also been established (21) that heme is rapidly excreted into the bile of rats when heme oxygenase is inhibited by Sn-protoporphyrin. We conclude that any transient cellular increases in heme concentration that may occur after Sn-protoporphyrin treatment do not have significant hematological consequences, even in animals, such as sph^{ha}/sph^{ha} mice, that are marginally viable.

This study confirms the nontoxic nature of Sn-protoporphyrin administered over a prolonged period of time and in very high doses to the sph^{ha}/sph^{ha} anemic mutant mouse, in which heme breakdown is far greater than that in man. It extends our earlier findings on Sn-protoporphyrin-mediated suppression of hyperbilirubinemia in genetically hemolytic anemic mice (14). On the basis of these and our previous findings, the Sn-protoporphyrin may prove to be a useful compound for the management of hyperbilirubinemia in selected clinical situations, since humans would require much smaller doses, e.g., 0.5–1.0 μmol/kg body weight (6), to suppress hyperbilirubinemia than those used in the present experiments.

Summary

The effects of long-term administration of very large doses of Sn-protoporphyrin on hematological indices, histological changes, plasma bilirubin levels, tissue heme oxygenase activity, and activities of heme biosynthetic enzymes, were examined in genetically anemic mutant mice with hemolytic anemia (sph^{ha}/sph^{ha}). Long-term weekly treatment with Sn-protoporphyrin (100 μmol/kg body weight for 32 wk) did not alter hematological indices, histological findings, or enzyme activities related to heme biosynthesis, even though it resulted in sustained decreases in microsomal heme oxygenase activity in the liver, kidney, and spleen, and a prolonged decrease in plasma bilirubin concentration. Inhibition of heme oxygenase did not alter the level of cytochrome P-450 in the liver and the kidney. The results indicate that long-term treatment with massive doses of Sn-protoporphyrin suppresses bilirubin formation but does not produce significant histopathological changes or appreciably interfere with heme synthesis, in this strain of genetically anemic mice. These findings provide further support for the idea that suppression of heme degradation to bile pigment by the inhibition of heme oxygenase may prove useful to the prevention of severe hyperbilirubinemia in humans.

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