Heme Biosynthesis and Drug Metabolism in Mice with Hereditary Hemolytic Anemia

HEMEE OXYGENASE INDUCTION AS AN ADAPTIVE RESPONSE FOR MAINTAINING CYTOCHROME P-450 IN CHRONIC HEMOLYSIS

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Mice homozygous for the nb (normoblastosis) gene have severe hemolytic anemia characterized by increased catabolism of hemoglobin. Heme biosynthesis and catabolism were measured in the tissues of homozygous (nb/nb), heterozygous (+/nb), and control (+/+) mice generated on the same genetic background. The functional capacity of the microsomal hemoprotein, cytochrome P-450, was also determined in the livers of these animals.

Mice homozygous for the nb gene defect had a marked increase in protoporphyrin content, δ-aminolevulinic acid (ALA)-dehydratase, and uroporphyrinogen I (URO)-synthase activities in erythrocytes. Lesser increases were observed in liver and spleen of nb/nb mice. The homozygous mice also had a marked increase in microsomal heme oxygenase activity in the liver, kidney, and spleen compared to normal controls. The increase in heme oxygenase activity is attributable to a higher specific activity per mg of microsomal protein in the case of the liver and the kidney and to the marked organomegaly in the case of the spleen.

Mice heterozygous for nb (+/nb) which do not have the overt hemolytic syndrome had normal levels of heme oxygenase activity, but elevated levels of ALA-dehydratase, URO-synthase, and protoporphyrin in blood. The increases observed in these indices in the heterozygotes were of a lesser magnitude than those observed with the nb/nb mice. The seemingly normal blood picture of the heterozygotes, therefore, probably reflects a compensated hemolytic state, as ALA-dehydratase, URO-synthase, and protoporphyrin levels are known to be increased in younger red cells.

These enzymes and protoporphyrin were marginally increased in the liver, kidney, and spleen of the +/nb mice; heme oxygenase activity in these tissues was not altered. Both the homozygotes (nb/nb) with extensive hemolytic anemia and the heterozygotes (+/nb) without overt hemolysis had normal levels of microsomal cytochrome P-450 and no impairment of ethylmorphine N-demethylase or benzo(a)pyrene hydroxylase activities in the liver. Cytochrome P-450 in the whole liver of the homozygotes was increased due to hepatomegaly. Levels of hepatic ALA-synthase were also comparable for both control and homozygous animals.

These data contrast with previous studies in which a single injection of hemin in experimental animals acutely inhibited increased synthesis of ALA-synthase and microsomal cytochrome P-450 in response to an inducing chemical, such as phenobarbital. Our results indicate that in the livers of homozygous (nb/nb) mice an adaptive response develops during chronic hemolysis which prevents excess free heme from repressing heme biosynthesis and the formation of microsomal cytochrome P-450.

A major fraction of the heme synthesized in the liver is utilized for the formation of microsomal cytochrome P-450 (1), a hemoprotein which is the terminal oxidase of the hepatic system of drug-metabolizing monooxygenases. These monooxygenases catalyze the metabolism of a variety of drugs, carcinogens, and other environmental chemicals, as well as endogenous substrates such as steroid hormones (2, 3). The functional capacity of these enzyme systems is affected by a variety of factors, including drugs, hormones (2), vitamins (4), metals (5), lipids, proteins, and carbohydrates (6). When heme is acutely administered to experimental animals, the synthesis of cytochrome P-450 is greatly suppressed (7, 8) and this effect is considered to be the result of inhibition of the biosynthesis of δ-aminolevulinic acid (ALA)-synthase (7), the first and rate-limiting enzyme for heme biosynthesis in the liver (9).

Since short term heme administration is known both to increase heme oxygenase (5) and to interfere with the formation of cytochrome P-450 (7), this study was undertaken to examine the effect of chronically increased heme production, due to hemolysis, on the levels of cytochrome P-450 and associated enzymic activities. Heme biosynthetic enzymes and the intermediary in the heme biosynthetic pathway, protoporphyrin, as well as the enzymes, the rate-limiting enzyme in heme catabolism, were also studied.

Mice homozygous for the nb (normoblastosis) gene (10) were used in these studies. The homozygous mice (nb/nb) have severe chronic hemolytic anemia and resultant marked elevation of 15CO production from 14C-labeled hemoglobin (approximately 30-fold) (11), increased levels of serum bilirubin (12), greenish urine, and increased activities of the enzymes involved in heme biosynthesis as well as of protoporphyrin concentration in their red blood cells (13). The levels of cytochrome P-450 and associated drug-metabolizing en-

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The abbreviations used are: ALA, δ-aminolevulinic acid; PBG, porphobilinogen; URO, uroporphyrinogen I.
zymes, heme pathway enzymes, and the intermediate, protoporphyrin, and heme oxygenase activity were determined in the liver, spleen, and kidney in the affected homozygous animals (nb/nb), in mice heterozygous for the nb gene defect but without overt hemolysis (+/nb), and in normal control animals (+/+) which were generated on the same genetic background.

MATERIALS AND METHODS

Mice—WBB6F1 female mice ranging in age from 7 to 9 months were used in this study. Mice homozygous for normoblastic anemia (nb/nb) (12 animals), heterozygous mice (+/nb) (6 animals), and normal controls (+/+) (12 animals) were reared in the research colonies at The Jackson Laboratory. Heterozygous mice had severe normocytic, hypochromic anemia manifest soon after birth and throughout their life span. Characteristic findings were low red cell counts (mean 4.9 x 106 cells/mm3), low hemoglobin values (mean 4.9 g/100 ml of blood), low hematocrit (mean 24%), high reticulocyte counts (50 to 60% of total erythrocytes), marked splenomegaly (splenic weight equal to 8 to 10% of body weight), hepatomegaly, and bone marrow hyperplasia characterized by normoblastic (erythroid) proliferation (10).

Heterozygous mice had normal red cell counts (mean 8.3 x 106 cells/mm3), hemoglobin (mean 13.7 g/100 ml), hematocrit (mean 43%), and reticulocyte counts (mean 4.8%) (14), although their 14C0 production was elevated compared with normal control mice. All of the mice used in this study were generated on the same genetic background by crossing heterozygotes (+/nb) maintained on two different genetic backgrounds (WB/Re and C57BL/6J). Mice were maintained on Jackson-Emory-Morse diet 96W. They were fed and watered ad libitum. The principles of laboratory animal care as made on freshly prepared specimens. Preparation of Microsomes—The homogenate (250 mg wet weight of organ/ml) was centrifuged at 9,000 x g for 20 min. The supernatant was removed and an aliquot was saved for benzo(a)pyrene hydroxylase determination. The remaining supernatant was centrifuged at 100,000 x g for 1 hr and supernatants were discarded. Pellets were resuspended to restore the original volume with a reaction mixture containing in micromoles/ml, of glycine (75), citrate (75), Tris buffer, pH 7.4 (37.5), phosphate buffer, pH 7.4 (37.5), MgCl2 (15), EDTA (7.5), and pyridoxal 5'-phosphate (22.2). Duplicates of 300-µl aliquots were incubated at 37°C for 80 min in a metabolic shaker at 60 oscillations/min. The mixture was terminated by the addition of 100 µl of ice cold 10% trichloroacetic acid. The reaction mixture was then centrifuged at 10,000 x g for 5 min, and the resulting microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.4). Each milliliter of the suspension contained 250 mg of organ in wet weight.

Protoporphyrin Assay—Protoporphyrin content was determined colorimetrically by the method of Sassa et al. (15) using 2 µl of whole blood or 5 µl of tissue homogenate.

ALA Dehydratase Assay—ALA-dehydratase activity was assayed colorimetrically using 5 µl of whole blood or tissue homogenate both in the absence and in the presence of 20 mM dithiothreitol (18). The assay without dithiothreitol reflects the enzyme activity present in the tissue in a functional form and the assay with dithiothreitol reflects the total enzyme activity of both endogenously active and dithiothreitol-activated forms.

URO-Synthase Assay—URO-synthase activity was determined fluorometrically using 2 µl of tissue homogenate as described previously (20) and the product formed was measured by the method of Nebert and Gelboin (21). The 9000 x g supernatant was used in this assay, since previous studies (22) had shown that the apparent Michaelis constant was similar whether the 9000 x g supernatant or microsomes were used.

Ethylmorphine N-Demethylase Assay—The composition of the incubation mixture for the N-demethylase assay was similar to that described previously (23), except that nicotinamide was omitted from the incubation mixture. Formaldehyde formed from the N-demethylase reaction was measured by the method of Nash (24) as modified by Anders and Mannering (25).

Cytochrome P-450 Determination—Cytochrome P-450 was determined on microsomal suspensions containing the equivalent of 100 mg of tissue wet weight/ml of 0.1 M phosphate buffer, pH 7.4. The hemeprotein content was determined by the method of Omura and Sato (26), using an Amino DW-2 spectrophotometer in the split beam mode. An extinction coefficient of 91 µm-1 cm-1 between 450 and 490 nm was used to calculate the concentration of hemoprotein.

Heme Oxygenase Assay—Microsomal heme oxygenase activity was determined using 30 mg of protein of 9000 x g supernatant fractions from kidney and spleen and 18 mg of protein using microsomes from liver. The assay was carried out according to the method of Tenhunen et al. (27). Bilirubin formation was calculated using an extinction coefficient of 40 nm-1 cm-1 between 468 and 530 nm (28).

RESULTS

Organ weights of control (+/+-), heterozygous (+/nb), and homozygous (nb/nb) mice are shown in Table I. No significant differences in liver, spleen, and kidney weights were observed between the +/+- and +/nb mice. In contrast, homozygous nb/nb mice had considerably larger livers and kidneys and an 18-fold increase in the mean weight of the spleens. The protein content of the 9000 x g supernatant from livers was (mean ± S.E.) 108 ± 8, 95 ± 2 and 118 ± 5 µg of protein/g of liver for +/+-, +/nb, and nb/nb mice, respectively, and there was no significant difference among genotypes. Microsomal protein content, although similar for the +/+- and +/nb mice (10.4 ± 0.4 and 16.3 ± 0.3 µg of protein/g of liver, respectively), was significantly higher for the nb/nb mice (29.5 ± 0.8 µg of protein/g of liver, p < 0.05).

Cytochrome P-450 and Associated Enzymic Activities of +/+-, +/nb, and nb/nb Mice—The hepatic cytochrome P-450 contents of +/+-, +/nb, and nb/nb mice are shown in Fig. 1. The cytochrome P-450 levels of the +/+- and +/nb mice were not significantly different whether expressed on a per g of

| Organ       | Organ weight | Organ weight | Organ weight |
|-------------|--------------|--------------|--------------|
| Livera       | 0.048 ± 0.001| 0.044 ± 0.002| 0.076 ± 0.003b |
| Spleena      | 0.005        | 0.005        | 0.093        |
| Kidneyb      | 0.0175       | 0.0112       | 0.0224       |

a Each value represents mean ± S.E. for livers from five mice.
b Value significantly different from liver weights of +/+- and +/nb mice (p < 0.05).

c Each value represents mean of tissues pooled from each genotype.

TABLE I

Organ weights of +/+-, +/nb, and nb/nb mice

| Organ       | +/+- | +/nb | nb/nb |
|-------------|------|------|-------|
| Liver       | 0.048 ± 0.001| 0.044 ± 0.002| 0.076 ± 0.003b |
| Spleen      | 0.005        | 0.005        | 0.093        |
| Kidney      | 0.0175       | 0.0112       | 0.0224       |
liver, per mg of microsomal protein, or on a per organ basis. On the other hand, nb/nb animals had a small but significant decrease in cytochrome P-450 content per mg of microsomal protein, although because of marked hepatomegaly, they had much greater levels of cytochrome P-450 per g of liver or per organ (Fig. 1).

The carbon monoxide difference spectra of liver microsomes of the +/+, +/nb, and nb/nb mice are shown in Fig. 2. The spectra of liver microsomes of both +/+ and +/nb mice showed a major peak at 450 nm and a minor peak at 420 to 422 nm with a trough at 430 nm. In contrast, the nb/nb microsomes had a considerably higher 420 nm peak. CO-hemoglobin probably contributes partially to the absorbance observed at this wavelength. If instead of the CO-difference spectrum the dithionite-induced difference spectrum was determined, the absorbance at 420 nm was markedly decreased, but could not be totally eliminated. The same cytochrome P-450 content was obtained by using either the CO-difference spectrum or the dithionite difference spectrum. As with microsomes from +/+ and +/nb mice, microsomes from nb/nb mice showed a major peak at 450 nm. As can be seen in Fig. 2, the base-line obtained on dithionite reduction of microsomes from nb/nb mice in sample and reference cuvettes was markedly distorted at the shorter wavelengths and could not be corrected. This distortion was not observed in other experiments when hemin, bilirubin, or biliverdin was added to microsomes from control mice. The base-line shift may, therefore, be due to other pigments, such as green pigments, present in nb/nb mice. In any case, the contribution of this shift in the baseline to the difference in absorbance between 450 nm and 490 nm used to calculate cytochrome P-450 levels was minimal.

The monooxygenase activities in livers of the various mice were proportional to their contents of cytochrome P-450 (Figs. 3 and 4). Ethylmorphine N-demethylase (Fig. 3B) and benzo(a)pyrene hydroxylase (Fig. 4B) activities were similar for all genotypes when the enzyme activities were expressed per g of liver. In contrast, when expressed per mg of microsomal protein, these enzymic activities were slightly lower in the nb/nb mice than in the +/+ or +/nb animals (Figs. 3A and 4A). As with cytochrome P-450, however, the nb/nb mice displayed a marked increase in both enzyme activities per organ which was related to hepatomegaly (Figs. 3C and 4C).

These results indicate that there are no significant differences in the microsomal cytochrome P-450 content or the monooxygenase activities of livers from +/+ and +/nb mice. The results also demonstrate that, although there is a slight reduction of cytochrome P-450 and associated monooxygenase activities per mg of microsomal protein in the nb/nb mice, these activities are not reduced when expressed on a per organ basis because of organomegaly. On the contrary, the levels of cytochrome P-450 and monooxygenase activities in the nb/nb mice were markedly increased when expressed on a per organ basis.
Heme Oxygenase Activities in Liver, Kidney, and Spleen of +/+, +/nb, and nb/nb Mice—The activity of heme oxygenase, the rate-limiting enzyme in the breakdown of heme to bilirubin, was determined in the liver, spleen, and kidney of the +/+ and +/nb mice and the data are shown in Fig. 5. The microsomal heme oxygenase activities in the three organs of the +/+ and +/nb mice were equivalent whether expressed on a per mg of microsomal protein, per g of organ, or per organ basis (Fig. 5). Strikingly different results, however, were observed with heme oxygenase activity in the nb/nb mice. In the liver, spleen, and kidney, the levels of heme oxygenase activities were approximately 10-, 25-, and 30-fold higher, respectively, than the levels observed in the +/+ and +/nb mice on a per organ basis (Fig. 5 C, F, and I). On the other hand, the heme oxygenase activity in the spleen was not significantly different in the +/+ and +/nb mice and expressed as nanomoles of bilirubin formed per mg of microsomal protein (Fig. 5G) or per g of spleen (Fig. 5H). Hepatic and renal heme oxygenase activities in the nb/nb mice were much higher than in the +/+ or +/nb mice (Fig. 5 A to F). Thus, the heme oxygenase activity in the liver, kidney, and spleen of the nb/nb mice was substantially increased over that of controls. This increase was principally due to a marked increase in the specific activity of the enzyme per mg of microsomal protein in the liver and kidney, although both organs increased approximately 50% in size as well. On the other hand, the striking increase in spleen weight (18-fold) accounted entirely for the increase in heme oxygenase activity of this organ.

Heme Pathway Enzymes and Protoporphyrin Content in +/+, +/nb and nb/nb Mice—The concentration of the heme pathway intermediate, protoporphyrin, was determined in erythrocytes, liver, spleen, and kidneys (Table II). The protoporphyrin concentration of the tissues was considerably higher in the nb/nb mice than in the +/+ or +/nb mice. Erythrocyte protoporphyrin levels in the +/nb mice were significantly higher than those in the +/+ mice. These results are consistent with our previous observations in the +/+ and in the +/nb mice (14). In contrast to erythrocyte protoporphyrin levels, there were no significant differences in protoporphyrin content of the liver, spleen, and kidney from the +/nb and +/+ mice.

ALA-dehydratase activity was determined both in the absence and the presence of dithiothreitol (Table III). ALA-dehydratase activity was much higher in the erythrocytes and spleen of nb/nb mice than in the +/nb or +/+ mice. No significant differences in ALA-dehydratase of tissues from the +/nb or +/+ mice were observed. ALA-dehydratase activity was greatly activated by the presence of dithiothreitol added in vitro to erythrocyte lysates but not in homogenates of other tissues (Table III). These results suggest that this enzyme is present in a fully active form in liver, kidney, and spleen; in red cells, however, this enzyme is present in a partially active form, possibly due to a limitation in sulfhydryl regenerating capacity of erythrocytes.

URO-synthase activity was markedly elevated in erythro-

![Fig. 4. Benzo(a)pyrene hydroxylase (OHBP) activity in the livers of +/+, +/nb, and nb/nb mice. Each bar represents the mean ± S.E. of five determinations with +/+ or nb/nb mice and three determinations with +/nb mice, two livers being pooled for each determination. Asterisk represents value which is significantly different from +/+ mice (p < 0.05).](http://www.jbc.org/)

![Fig. 5. Heme oxygenase activity in the liver, kidneys, and spleens of +/+, +/nb, and nb/nb mice. Heme oxygenase activity is expressed as nanomoles of bilirubin formed per mg of microsomal protein, or per g of tissue, or per organ. Liver (A, B, and C): each bar represents the mean ± S.E. of five determinations with +/+ or nb/nb mice and three determinations with +/nb mice, two livers being pooled for each determination. Kidney (D, E, and F): all kidneys from each group were pooled and samples were analyzed in quadruplicate. Spleen (G, H, and I): with +/+ and +/nb mice, all spleens in each group were pooled and samples were analyzed in quadruplicate. Spleens from nb/nb mice were analyzed individually and the bars represent the mean for five spleens from nb/nb mice.](http://www.jbc.org/)

| Tissue          | +/+          | +/nb         | nb/nb         |
|-----------------|--------------|--------------|---------------|
| Protoporphyrin content (µg/100 ml red blood cells) |              |              |               |
| Erythrocytes    | 107 ± 3 (10) | 170 ± 9 (6)* | 1029 ± 20 (10)* |
| Spleen          | 5.13         | 7.75         | 37.86         |
| Kidney          | 3.78         | 4.72         | 10.54         |

*Values statistically different from the +/+ value (p < 0.01).
erythrocytes, liver, spleen, and kidney of nb/nb mice when compared to the control (+/+). The ALA-synthase activity of erythrocytes, liver, and kidney was slightly higher in the +/nb mice than in the +/+ mice (Table IV). The ALA-synthase activity of liver in the control (+/+ and in the homozygous (nb/nb) mice is shown in Table V. The homozygous anemic mice displayed ALA-synthase activity approximately 20% higher than that observed in the control mice on a per g of liver or per mg of protein basis. The total ALA-synthase activity in the liver was approximately 50% higher in the nb/nb mice than in the +/+ mice.

**DISCUSSION**

The results of this study clearly demonstrate that the levels of microsomal cytochrome P-450, and certain associated enzymic activities, such as ethylmorphine N-demethylase and benzo(a)pyrene hydroxylase, were not impaired in the livers of nb/nb mice under conditions of severe chronic hemolytic anemia and continuous excess production of heme and bilirubin. Erythrocyte survival in the nb/nb animals is less than 2 days as compared to the normal value of 60 days and there is a resultant marked elevation of the enzymes involved in heme biosynthesis and in protoporphyrin of the red cells (13) due to an increase in young reticulocytes in the circulating blood. The amount of heme released during red cell breakdown in an nb/nb mouse would be approximately 2.5 µmol/day, assuming the total blood volume and hemoglobin content of such a mouse to be 1.65 ml/30 g of body weight and 4.9 g/dl, respectively (10). If this concentration of heme (equivalent to 1.5 µmol/day) were presented directly to the hepatic microsomes engaged in the formation of cytochrome P-450, it might be expected to markedly repress the formation of this hemoprotein, since concentrations of heme as well as hemoglobin of 10^{-7} M are known to inhibit the synthesis of ALA synthase, the rate limiting enzyme of the heme biosynthetic pathway in cultured chick embryo liver cells (30). The inhibiting concentration of hemin on ALA-synthase formation in cultured mouse liver cells is not known. However, Marver (7) and Schacter et al. (8) reported that hemin administration (2.5 µmol/100 g of body weight) blocks the increase of microsomal cytochrome P-450 and cytochrome b5 induced in the liver by treatment of rats with phenobarbital. The slight reduction we observed in cytochrome P-450 and associated monooxygenase activities in the liver, when expressed on a per mg of microsomal protein basis, might be to some extent due to such a hemin effect on the synthesis of the microsomal cytochromes. However, it appears to be best explained by an increased number of Kupffer cells in response to the red cell breakdown (31) in these mice. While both hepatocytes and Kupffer cells are rich in endoplasmic reticulum and sediment as the microsomal pellet, cytochrome P-450 and associated monooxygenases are present in higher concentrations in the hepatocytes rather than in the Kupffer cells (32). Accordingly, a relative increase in Kupffer cells over hepatocytes would be expected to produce an apparent reduction in the specific activities of the mixed function oxidases per mg of microsomal protein which was in fact observed.

In mice with normoblastosis, an enhanced release of heme from hemoglobin was associated with a marked induction of microsomal heme oxygenase activity, the enzyme which metabolizes free heme to biliverdin, which is in turn reduced to bilirubin and excreted as conjugates in the urine.
bile pigment by the cytosolic enzyme biliverdin reductase (27). Additional evidence for increased heme catabolism in these mutants is an elevated serum bilirubin level, excretion of greenish urine, a 20-fold increase in fecal urobilinogen (12), as well as a 30-fold increase in CO production which derives from carbon in the α-methene bridge of heme (11). Heme oxygenase and cytochrome P-450 are both known to be present in the microsomal fraction of hepatocytes and the induction of heme oxygenase is considered to bring about a decrease of cytochrome P-450 (5); however, our results clearly demonstrate that an increased level of heme oxygenase activity as a result of chronic hemolysis does not in itself result in markedly reduced levels of the heme protein cytochrome P-450. This finding suggests that a compensatory mechanism develops to prevent the repression by heme of heme biosynthesis and cytochrome P-450 formation in chronic hemolysis. This compensatory mechanism is most likely attributable to the induced heme oxygenase itself which by degrading excess free heme prevents it from exerting a repressive action on the biosynthesis of heme. Alternatively, heme derived from senescent red cells is metabolized entirely in Kupffer cells, thus freeing the hepatocytes from its inhibitory action.

These results are consistent with the previous findings in human subjects that normal rates of drug metabolism occur in children with homozygous β-thalassemia (33) or with sickle cell anemia (34). In such patients with chronic severe hemolysis, there is no evidence of impairment in cytochrome P-450-mediated drug metabolism (33, 34); more importantly, there is no impairment of inducibility of oxidative drug metabolism by the drug phenobarbital (34). Our results further indicate that the normal levels of drug metabolism in mice with chronic hemolytic anemia are maintained even in the presence of markedly induced microsomal heme oxygenase activity.

Total heme oxygenase activity per spleen in the nb/nb mice was approximately 2-6-fold greater than in the liver and the kidney, respectively (Fig. 5C). In contrast, in the +/+ and +/nb mice, the total heme oxygenase activity in the liver (Fig. 5C) was greater than in the spleen (Fig. 5F). These observations indicate that in normal animals, although the spleen has a higher specific activity of heme oxygenase than the liver, the liver is the major site of heme destruction because of its greater size. On the other hand, in mice with severe hemolytic anemia, the spleen becomes the major site of heme destruction because of its marked increase in size and in total heme oxygenase activity. In the spleen, the level of microsomal heme oxygenase activity per mg of microsomal protein is similar for +/+ and +/nb, and nb/nb animals (Fig. 5G). These observations are consistent with the findings of Tenhunen et al. (35) that phenylhydrazine-induced hemolysis did not significantly increase heme oxygenase activity per gram of spleen, but increased the total heme oxygenase activity of this organ due to splenomegaly. Schacter et al. (36) also reported that although total heme oxygenase activity was significantly increased in the spleens of human patients with chronic hemolytic anemia, the enzyme activity per mg of microsomal protein was not significantly increased when compared to patients with normal spleens.

In the nb/nb mice the increase in the activity of splenic heme oxygenase resulted primarily from an 18-fold increase in the size of the organ. However, in the liver and kidney of these animals, the increase in heme oxygenase activity resulted from marked increases in the specific activity of the enzyme (Fig. 5, A and D). It is also of interest that in the control (+/+ and the heterozygous +/nb) mice, the specific activity of heme oxygenase was considerably higher in the spleen than in the liver and kidneys of the same animals. In fact, the increased enzyme activity in the liver of the nb/nb mice was only twice as high and in the kidneys was approximately the same as the enzyme activity in the spleen. Accordingly it can be concluded that heme oxygenase activity in the spleen is constitutively induced in the control animal and the total splenic enzyme activity can increase only as a result of an increase in the number of cells in this organ. No significant stimulatory effect on splenic heme oxygenase activity was also observed in rats treated with cobalt, a metal which markedly induces the enzyme in liver (5, 37). In contrast, in the homozygous (nb/nb) mice, the liver and kidneys respond to hemolysis mainly by increasing the specific activity of the enzyme and less by proliferating cellular elements.

Mice heterozygous for normoblastosis (+/nb) showed significantly higher levels of erythrocyte protoporphyrin, ALA-dehydratase, and URO-synthase activities in comparison to the normal (+/+). Their red cell survival time was also slightly shorter than normal, as indicated by an increased production of 14CO from 14C-labeled hemoglobin. These results suggest that the seemingly normal hematological picture of the +/nb mice is in fact maintained by increased erythropoiesis which in turn is reflected by enhanced activity of two of the enzymes that participate in heme biosynthesis. There were no significant alterations in the tissue levels of protoporphyrin and the heme pathway enzymes studied in the +/nb mice. There were also no changes in the levels of cytochrome P-450 and associated monooxygenase activities. Since tissue heme oxygenase activities in the +/nb mice were identical with those of control mice, it is also clear that a slight increase in free heme derived from hemoglobin catabolism can be oxidized without increasing the level of heme oxygenase activity.

Hypothetical schemes illustrating the possible differences between the acute and chronic effects of heme on heme biosynthesis and P-450 are shown in Fig. 6. As discussed previously, acute administration of excess heme represses
drug-mediated induction of ALA-synthase and microsomal cytochrome P-450 (7, 8) (Fig. 6A). In contrast to these acute effects of heme, the results of the present study demonstrate that the long term increase in heme production due to chronic hemolysis does not lead to an inhibition of hepatic heme biosynthesis or a repression of microsomal cytochrome formation (Fig. 6B). It is particularly important to note that hepatic ALA-synthase in the homozygous (nb/nb) mice is not depressed compared with that in the control mice (Table V). Since hepatocytes are exposed to a greater concentration of heme in the nb/nb mice, it must be assumed that an adaptive mechanism develops during long term hemolysis which counteracts the ability of heme to repress its own synthesis and to diminish cellular cytochrome P-450 content. It is evident that the markedly increased level of microsomal heme oxygenase must be a critical element in this adaptive response since increased heme oxygenase by catabolizing heme would prevent excess free heme from exerting these repressive biological actions.

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