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# Release of Free, Redox-active Iron in the Liver and DNA Oxidative Damage Following Phenylhydrazine Intoxication

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**ABSTRACT.** Following the subchronic intoxication of rats with phenylhydrazine, resulting in marked anemia, reticulocytosis, methemoglobinemia and increased hemocatheresis, the hepatic content of total iron was increased, as was hepatic ferritin and its saturation by iron. A striking increase (approximately 7-fold) was also observed in free iron which appeared to be redox-active. The increase in liver free iron involved the hepatocellular component of the liver. Since DNA is one of the cellular targets of redox active iron, liver DNA from phenylhydrazine-treated rats was analyzed by electrophoresis and found to be markedly fragmented. Experiments with isolated hepatocytes in culture or in suspension challenged with phenylhydrazine or Fe-nitrilotriacetate strongly suggested that the DNA damage was due to reactive iron rather than to the hepatic metabolism of phenylhydrazine. The levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), a specific marker of oxidative DNA damage, were significantly higher in phenylhydrazine-treated rats as compared to untreated controls. The prolongation of phenylhydrazine treatment over a period of 6 weeks resulted in a persistent damage to DNA and in phenotypic changes such as an increase in hepatocyte γ-glutamyl transpeptidase (γ-GT, EC 2.3.2.2) activity. Possible relationships between iron overload, iron release, DNA damage and tumor initiation are discussed. BIOCHEM PHARMACOL 53;11:1743–1751, 1997. © 1997 Elsevier Science Inc.

**KEY WORDS.** Phenylhydrazine; free iron; liver; DNA fragmentation; 8-oxo-7,8-dihydro-2′-deoxyguanosine; γ-glutamyl transpeptidase

Living cells require well-defined amounts of iron for survival, replication and expression of differentiated products [1]. Normally iron is transported and kept in specific proteins (transferrin, ferritin, lactoferrin and heme proteins), which prevents or minimizes its reaction with reduced oxygen derivatives. When released from these complexes in a so-called free form, iron is redox active and can promote, through the Fenton reaction, the formation of harmful oxygen species such as the hydroxyl radical [2–10]. Iron toxicity is most likely accomplished by an oxidative stress induced by free radical reactions primed by redox-active iron [11, 12]. The liver is the main site of iron storage; in iron storage diseases, such as hereditary hemochromatosis, the liver is primarily involved in iron overload, leading to hepatic cirrhosis and hepatocellular carcinoma [11].

Several models of hepatic iron overload have been developed, based on either the iron enrichment of the diet [13, 14] or the parenteral administration of iron in the form of various complexes, such as iron dextrane [15, 16] or iron nitrilotriacetate [17].

Hemolytic anemias, in particular hemoglobinopathies, have long been known to be associated with excessive deposition of iron in the liver and other organs. Among other hemolytic agents, phenylhydrazine is known to induce oxidative damage of both hemoglobin [18–20] and erythrocyte membrane proteins, the latter resulting in the formation of a neoantigen (senescent cell antigen) which is recognized by autologous IgG [21, 22]. These antibodies bind to erythrocytes and prime their removal from the circulation by cells of the reticulo-endothelial system, which accomplishes a massive hemocatheresis. The metabolism of iron in the reticulo-endothelial cells is believed to be similar to that occurring in the hepatocytes [11], the main difference being that the former take up iron as a result of their phagocytosis of senescent erythrocytes, rather than in soluble form (transferrin and non-transferrin

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*Abbreviations:* 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DFO, desferrioxamine; dGuo, 2′-deoxyguanosine; DTT, dithiothreitol; γ-GT, γ-glutamyl transpeptidase; MDA, malonaldehyde; TBARS, thiobarbituric acid-reactive substances.

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bound). Iron is then released from the reticulo-endothelial cells and transported by transferrin to other tissues (bone marrow, liver etc.). In phenylhydrazine-treated animals, an accelerated iron redistribution to various tissues can thus be anticipated.

The present study shows that the subchronic administration of phenylhydrazine to rats, commonly used to obtain reticulocytosis [23], resulted in a model of endogenously induced hepatic iron overload. Hepatic total iron was increased and, more importantly, the level of free iron was dramatically increased. We have previously shown [24, 25] that in erythrocytes incubated with a number of oxidizing agents (phenylhydrazine, divicine, isouramil, etc.) iron is released from hemoglobin in a free (desferrioxamine-chelatable) form, and that this free iron is capable of inducing oxidative reactions (lipid peroxidation and protein oxidation) in the cell membrane. We have also shown [26] that the released iron is capable of inducing oxidative changes in erythrocyte membrane proteins which appear to be related to the generation of senescent antigen. In the present work, we studied the effects of iron overload and of free iron on selected molecular targets within the hepatocytes. Since the ability of redox active iron to interact with DNA has been widely demonstrated [27–30], we investigated whether DNA damage occurs in this model of hepatic iron overload. The results indicate that liver DNA is oxidatively damaged in phenylhydrazine-treated animals. In view of the above-mentioned relationships between iron overload and hepatocarcinogenesis, we also investigated whether signs of tumor initiation could be detected; an increased expression of γ-glutamyl transpeptidase was observed.

## MATERIALS AND METHODS

### *Chemicals*

Desferrioxamine (DFO) was kindly supplied by Ciba-Geigy (Basel, Switzerland). [$^{59}$Fe]$SO_4$, specific activity 32.89 mCi/mg, was from Dupont (Dreieich, Germany). Molecular weight marker-DNA was from U.S.B. (Cleveland, OH, U.S.A.). Phenylhydrazine hydrochloride was from Carlo Erba (Milan, Italy). RNase A from bovine pancreas was from Sigma (St. Louis, MO, U.S.A.). Collagenease type A, used for the preparation of isolated hepatocytes, was from Sigma. The solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade.

### *Animal Treatment*

Male Sprague–Dawley rats (Nossan, Correzzana, Milan, Italy) maintained on a commercial diet (Nossan) and weighing approximately 250 g were used. Phenylhydrazine, dissolved in saline and brought to neutral pH, was injected subcutaneously at the dose of 0.1 mmol/kg body wt. Control animals were treated with an equal volume of saline. In general, phenylhydrazine was administered for 6 days; the animals were not treated on the 7th and 8th days and sacrificed on the following day. Variations of the treatment period are reported in pertinent Tables and Figures. In some experiments the rats were fed a standard diet supplemented with 2.5% (w/w) carbonyl iron for 3 or 6 weeks.

### *Determination of Total Iron, Free Iron and Ferritin*

Blood samples were withdrawn from the abdominal aorta. The liver was perfused with 20 mL of cold saline and then homogenized (25% w/v) in 150 mM NaCl, 10 mM Tris-maleate buffer, pH 7.4. Total iron was measured by atomic absorption (flame ionization) (Varian Spectra AA 10 Spectrometer, Victoria, Australia) after acid digestion of the sample with 2 vol of concentrated $HNO_3$ and 1 vol of concentrated $H_2SO_4$ at 100°C for 30 min. A calibration curve was performed with $FeSO_4$. Free iron was measured using another aliquot of the homogenate to which DFO (25 μM) was added. After centrifugation of the homogenate at 2,000 × *g* (10 min) and then at 100,000 × *g* (30 min), the post-microsomal supernatant fraction was ultrafiltered in Centriflo® CF 25 cones (Amicon, Beverly, MA, U.S.A.). Determination of the DFO-iron complex (ferroxamine) was performed by HPLC according to Kruck *et al.* [31], with a number of modifications, as reported in detail in [32]. Ferritin was prepared from the supernatant fraction obtained by centrifuging the homogenate at 2,000 × *g* for 10 min. The preparation was performed by heating of sample and subsequent precipitation with ammonium sulfate according to the method of Penders *et al.* [33]. The prepared ferritin protein was quantified by the method of Lowry *et al.* [34]; ferritin iron was measured by atomic absorption after dissolving the ferritin protein in 32.5% (w/v) $HNO_3$ (50 μg/mL). Total and free iron in the spleen were measured as for the liver.

### *Lipid Peroxidation of Liver Preparations*

Liver homogenates (10% w/v) were prepared from controls and phenylhydrazine-treated rats as reported above. After centrifugation (40 × *g*, 5 min), the supernatant fractions were incubated for 60 min at 37°C. The thiobarbituric acid-reactive substances (TBARS) formed were determined [35] as an index of lipid peroxidation and referred to as moles of malonyldialdehyde (MDA) using an MDA standard.

### *Preparation of Liver DNA for Agarose Gel Electrophoresis*

Liver DNA was prepared from nuclei as follows. A portion of liver perfused with saline as above was homogenized (25% w/v) in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl, 0.25 M sucrose, 0.5 mM dithiothreitol (DTT) and 1 mM EGTA. The homogenate was centrifuged at low gravity (40 × *g*, 5 min). The supernatant fraction was used for the preparation of the nuclei essentially as reported by Bresnick *et al.* [36].

The nuclei corresponding to 1 g liver were resuspended with 20 mL of lysis solution, consisting of 75 mM NaCl, 5 mM EGTA, 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.5% (v/v) Triton X 100, pH 9.0. Fifty μL of an RNase A (Sigma) solution (10 mg/mL) were added and the suspension was incubated at 37°C for 30 min. DNA was extracted by adding an equal volume of 85% (v/v) phenol, pH 7.0, to the suspension and by following the method described by Sambrook *et al.* [37]. DNA was adjusted spectrophotometrically at the concentration of 50 μg/mL, analyzed on agarose gel electrophoresis as reported [37] and detected with ethydium bromide under UV light [37].

In some experiments, DNA was prepared directly from the liver homogenate as follows. The perfused liver was homogenized (25% w/v) in 10% Tris-maleate buffer, pH 7.4. The homogenate was centrifuged at 2000 × *g* for 15 min and the pellet was resuspended in the homogenization buffer. An aliquot of the resuspension (equivalent to 1 g liver) was added to the lysis solution used above. After the addition of 50 μL of the RNase A solution, the mixture was incubated and DNA was extracted with phenol as reported for the isolated nuclei.

### *Preparations of Liver DNA for Determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo)*

Isolation and purification of liver DNA were performed according to Fiala *et al.* [38]. DNA was solubilized in 1 mL of 20 mM acetate buffer, pH 4.8, and denaturated at 90°C for 3 min. To an aliquot of resuspended DNA (150 μL), 20 μL of P1 nuclease (5 IU) were added, and the mixture was incubated at 37°C for 1 hr in the dark. At the end of the incubation, the mixture was digested for 1 hr at 37°C with 10 μL alkaline phosphatase (3 IU) in the presence of 80 μl Tris-HCl buffer, pH 7.8. All samples were protected from light with aluminium foil in order to avoid *in vitro* artifactual oxidation. The hydrolysed mixture was then centrifuged and 70–80 μL of the supernatant were injected into an HPLC apparatus. The separation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and 2′-deoxyguanosine (dGuo) was performed with an LC/9A Shimatzu HPLC pump (Tokyo, Japan) equipped in series with a UV detector to measure the levels of dGuo and with a Coulochem detector (ESA model 5100 with a 5010 analytical cell, Bedford, MA, U.S.A.) to measure the levels of 8-oxodGuo. Two C18 reverse-phase columns in series (Supelco, Bellefonte, PA, U.S.A., 5 μm, i.d. 0.46 × 25 cm) were used. The eluting solution was $H_2O/CH_3OH$ (85:15, v/v) with 50 mM $KH_2PO_4$, pH 5.5, at a flow rate of 0.68 mL/min. The potentials set for electrodes 1 and 2 of the electrochemical detector were 0.02 and 0.5 V, respectively; the UV detector was set at 245 nm. The detectors were connected to a Shimatzu integrator for the determination of peak areas. The retention time for dGuo was 12.5 min and for 8-oxodGuo 18.4 min. The levels of 8-oxodGuo were expressed as the ratio of 8-oxodGuo relative to $10^5$ dGuo bases and calculated using the peak areas of 8-oxodGuo and dGuo relative to the standards. We prepared 8-oxodGuo as chromatographic standard following the method of Kasay and Nishimura [39] as reported elsewhere [40], while dGuo was obtained from Sigma (Sigma-Aldrich, Milan, Italy).

### *Experiments with Isolated Hepatocytes in Culture or in Suspension*

Isolated hepatocytes were prepared as reported [41] and washed three times with 0.15 M PBS. The cells were resuspended in the culture medium (Waymouth MB 752/1, Sigma), plated and incubated for 4 hr at 37°C in 95% $O_2$, 5% $CO_2$. The non-adhering cells were removed by three washings with the culture medium. After the addition of fresh medium the cells were incubated for 6 days in the presence of either 50 μM phenylhydrazine or 50 μM Fe-nitrilotriacetate (prepared as reported [42] or the vehicle only (controls). The medium to which drugs were added was changed daily. At the end of the incubation period, the cells were lysed with the lysis solution used above, and the DNA was extracted by the phenol method [43] and analyzed on agarose gel electrophoresis [37] as reported above.

In the experiments with isolated hepatocytes in suspension, the hepatocytes were prepared as above [41] and resuspended in Hank's buffer at a concentration of 10 × $10^6$ cell/mL. They were incubated at 37°C for 2 or 3 hr in 95% $O_2$, 5% $CO_2$. Phenylhydrazine (50 μM) was added to the treated samples. DNA was extracted and analyzed as above.

### *Experiments with Rats Injected with [$^{59}$Fe]-labelled Erythrocytes*

Labelled erythrocytes were obtained from iron-deficient animals, to which labelled ferritin was injected i.v. Iron deficiency was induced by feeding the rats (150–170) an iron-restricted diet (Altromin C1038, 6.3 μg Fe/g, Altromin, Lage, Germany) for 40 days. Three iron-deficient rats were injected intravenously with 500 μg of $^{59}$Fe-labelled ferritin (total radioactivity 84.843 × $10^6$ cpm) dissolved in 0.5 mL Hepes buffer 0.5 M, pH 7.5. Labelled ferritin was obtained as reported by Treffry and Harrison [44]. [$^{59}$Fe] incorporation into erythrocytes was tested both 24 and 48 hr after [$^{59}$Fe]-ferritin injection (blood was withdrawn from the tail vein) and was more than 20 and 50% of the injected dose at 24 and 48 hr, respectively, assuming a total blood volume of 10 mL for a rat of 250–270 g. [$^{59}$Fe]-labelled erythrocytes were then prepared from the rats 48 hr after ferritin injection, and one mL of packed cell (total radioactivity 10.832 × $10^6$ cpm) was injected intravenously into normal rats. One half of the animals were treated with phenylhydrazine as described above, while the other half served as controls. All the animals were sacrificed on the 9th day, and blood, liver and spleen were withdrawn. The radioactivity in whole blood

**TABLE 1. Liver and spleen total and free iron and liver ferritin in rats treated with phenylhydrazine for 6 days or with saline (controls)**

| | Liver total iron (nmol/g) | Liver free iron (nmol/g) | Liver ferritin (pmol/g) | Ferritin iron (mol Fe/mol ferritin) | Spleen total iron (nmol/g) | Spleen free iron (nmol/g) |
|---|---|---|---|---|---|---|
| Controls | 1628 ± 210 (13) | 7.15 ± 0.30 (4) | 50.0 ± 10.2 (7) | 1384 ± 97 (4) | 2348 ± 173 (3) | 7.6 ± 0.1 (4) |
| Phenylhydrazine | 2741 ± 241* (13) | 50.07 ± 7.40† (11) | 92.7 ± 14.7‡ (9) | 2380 ± 165† (4) | 6470 ± 382 (7) | 19.5 ± 2.2* (10) |

Phenylhydrazine was given s.c. at the dose of 0.1 mmol/kg body weight for 6 days. The animals were kept without treatment on the 7th and 8th days. They were sacrificed on the 9th day. Results are means ± S.E.M. The number of animals is reported in brackets.

* Significantly different from control, $P < 0.01$.

† Significantly different from control, $P < 0.001$.

‡ Significantly different from control, $P < 0.05$.

was 360 ± 0.5 × $10^3$ and 195 ± 3.5 × $10^3$ cpm/mL in control and phenylhydrazine-treated rats, respectively. Liver and spleen were homogenized as reported above, and the homogenates were centrifuged at 100,000 × *g* for 30 min. The supernatant fraction (soluble fractions) were ultrafiltered as reported above, and the radioactivity was measured in the whole homogenate, in the soluble fraction and in the ultrafiltered soluble fraction; the latter radioactivity was assumed to be that associated with free iron.

### *Histochemical Examination of the Liver for γ-Glutamyl Transpeptidase (γ-GT)*

γ-GT activity was revealed histochemically in acetone-fixed cryostat sections of liver tissue (thickness: 15 μ), using L-glutamic acid γ-(4-methoxy-β-naphthylamide) as substrate, according to the procedure of Rutenburg *et al.* [45]. Distribution of stain was assessed in selected sections counterstained with Janus green. Additional sections were post-fixed in diethylether/ethanol, 1/1, and processed for routine histology (haematoxylin-eosin).

### *Other Determinations*

Ig binding to erythrocytes was measured by radio-immunoassay with [$^{125}$I]-protein A as reported elsewhere [26]. Methemoglobin was determined as reported [24].

## RESULTS

The subchronic intoxication of rats with phenylhydrazine resulted in a decrease in the hematocrit level (47 ± 1.9 vs 54.5 ± 0.5 in controls) with marked reticulocytosis (82%) and methemoglobinemia (6.2 ± 0.8 μmol/mL vs 0.7 ± 0.1 in controls); a marked increase in spleen weight (1.5 ± 0.1 vs 0.3 ± 0.05 g/100 g body wt. in controls) was also observed. Erythrocytes from phenylhydrazine-treated animals, obtained at 48 hr after the beginning of the treatment, showed a significantly higher (44% increase) level of bound autologous IgG as compared to erythrocytes from controls, suggesting that senescent antigen had already formed on their surface. Table 1 shows that the hepatic content of total iron was increased (68%); hepatic ferritin was also increased (85%) as was its iron saturation (72%). Interestingly, a striking increase (7-fold) in free iron also occurred in the liver. Increased free iron was also found in the spleen, but such an increase was much lower than that in the liver (Table 1), indicating that the increase in free iron was not a mere consequence of the increase in total iron, which was higher in the spleen (275%) than in the liver.

In rats injected with $^{59}$Fe-labelled erythrocytes (see Materials and Methods), it was shown—as expected—that the increase in liver and spleen iron derives from erythrocytes. In fact, following phenylhydrazine treatment, a much higher radioactivity was detected in both liver and spleen ultrafiltered soluble fractions than in controls (see Table 2).

In order to ascertain whether the observed increase in iron levels indeed involved the hepatocellular component of tissue, isolated hepatocytes were prepared from the livers of control and phenylhydrazine-treated rats, and the level of free iron was measured. Such a level was higher in the hepatocytes from the phenylhydrazine-treated animals (9.7 μM vs 3.5 μM in controls).

Subsequent studies were dedicated to investigating whether such a massive presence of free iron could induce oxidative reactions in the liver. Table 3 shows that upon

**TABLE 2. Radioactivity in liver and spleen ultrafiltered soluble fractions from rats injected with $^{59}$Fe-labelled erythrocytes and then treated with phenylhydrazine for 6 days or with saline (controls)**

| | Liver (cpm per g equivalent of ultrafiltered soluble fraction) | Spleen (cpm per g equivalent of ultrafiltered soluble fraction) |
|---|---|---|
| Controls | 240 | 280 |
| Phenylhydrazine | 2,025 | 1,490 |

Iron-deficient rats were injected i.v. with ferritin containing $^{59}$Fe (see Materials and Methods). $^{59}$Fe-labelled erythrocytes were withdrawn and injected i.v. into normal rats. One half of the animals were treated with phenylhydrazine as reported in Table 2, while the other half served as controls. At the end of the treatment period, the animals were sacrificed and the radioactivity was measured in liver and spleen ultrafiltered (protein-free) soluble fractions.

**TABLE 3. *In vitro* lipid peroxidation of liver preparations (post-nuclear supernatant fractions) from phenylhydrazine-treated rats and respective controls**

| | MDA (nmol/g equivalent liver) |
|---|---|
| Controls | 5.9 ± 0.7 (6) |
| Phenylhydrazine | 18.2 ± 0.8* (6) |

Phenylhydrazine treatment was performed as reported in Table 1. Liver homogenates were prepared and centrifuged, and the supernatant fractions were incubated as reported in Materials and Methods. The amount of TBARS formed was determined and referred to as moles of malonaldehyde (MDA). Results are means ± SEM; the number of animals is reported in brackets.

* Significantly different from control, $P < 0.001$.

incubation of post-nuclear supernatants from phenylhydrazine-treated rats, a higher production of the lipid oxidation product, malonaldehyde, was obtained, an indication that free iron present as a result of phenylhydrazine treatment is redox-active.

Since, as previously mentioned, DNA is one of the cellular targets of redox cycling active iron, we then investigated whether any damage to liver DNA could be detected. As shown in Fig. 1, the DNA from phenylhydrazine-treated animals was fragmented, as evidenced by agarose gel electrophoresis. Such a fragmentation appears quite aspecific and not in the ladder-like pattern typical of apoptosis. The possibility that DNA damage was due to interactions with reactive metabolites of phenylhydrazine itself was tested by incubating isolated hepatocytes with phenylhydrazine both in suspension and in culture, and by analyzing their DNA. No DNA fragmentation was observed in either instance (Fig. 2). As a positive control, cultured hepatocytes were treated with ferric nitrilotriacetate; a clear DNA fragmentation was seen in these hepatocytes (Fig. 2).

In additional experiments, the levels of hepatic free iron

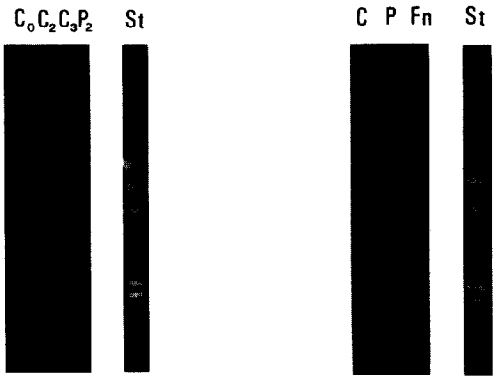


FIG. 2. Agarose gel electrophoresis of DNA from isolated hepatocytes treated with phenylhydrazine either in suspension (left) or in culture (right). Suspended or cultured hepatocytes were incubated with 50 μM phenylhydrazine for 2 hr or 6 days, respectively, as reported in Materials and Methods. A sample of cultured hepatocytes was also treated with 50 μM Fe-nitrilotriacetate (Fn). Electrophoresis was performed as in Fig. 1. Left panel: $C_0$, control zero time sample; $C_2$, control incubated without phenylhydrazine for 2 hr; $C_3$, control incubated without phenylhydrazine for 3 hr; $P_2$, incubated with phenylhydrazine for 2 hr; St, DNA standards. Right panel: C, control incubated without additions for 6 days; P, incubated with phenylhydrazine for 6 days; Fn, incubated with Fe-nitrilotriacetate for 6 days; St, DNA standards (for molecular size see Fig. 1).

were measured at different times after phenylhydrazine treatment (Fig. 3). When the animals were treated for 3 days only (instead of the usual 6 days), free iron, although increased, was still relatively low (Fig. 3, see legend). No DNA damage was seen in these animals (Fig. 3). On the other hand, when 3-day-treated animals were kept without treatment for an additional 5 days, in two cases (rats no. 1 and 2) the increase in free iron was much higher (Fig. 3, see legend) and DNA fragmentation was evident; on the other

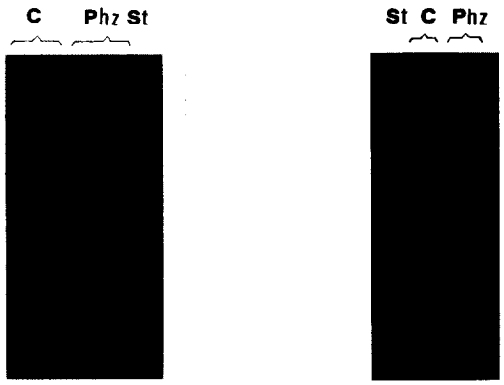


FIG. 1. Agarose gel electrophoresis of liver DNA from phenylhydrazine-treated rats and respective controls. Phenylhydrazine treatment was performed as reported in Table 1. Liver DNA was prepared from either the whole tissue (left) or the isolated nuclear fraction (right) as reported in Materials and Methods. Electrophoresis was performed on 0.8% agarose gel and DNA was revealed with ethydium bromide under UV light. C, controls; Phz, phenylhydrazine-treated rats; St, DNA standards (from top to bottom: 23, 9.4, 6.5, 4.4, 2.3, 2.0 Kbase-pairs).

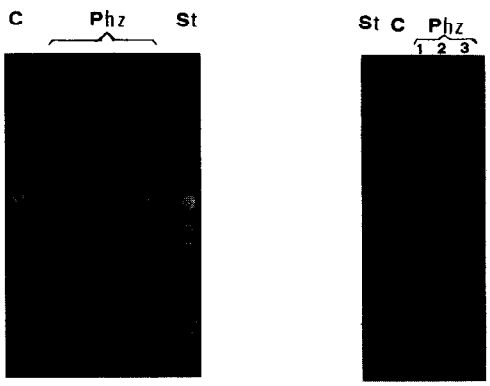


FIG. 3. Agarose gel electrophoresis of liver DNA from rats treated with phenylhydrazine for 3 days and sacrificed either on the 5th day (left) or the 9th day (right). The dose of phenylhydrazine was as reported in Table 1. Liver free iron was (nmol/g) 9.23 ± 0.88 on the 5th day and 65.5 (rat no. 1), 51.6 (rat no. 2) and 26.4 (rat no. 3) on the 9th day. Liver free iron in control animals was 5.0 ± 0.8 (nmol/g). C, controls; Phz, phenylhydrazine-treated rats. St, DNA standards (for molecular size see Fig. 1).

**TABLE 4. Levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) in liver DNA from phenylhydrazine-treated rats as compared to controls**

| | 8-oxodGuo/$10^5$ dGuo |
|---|---|
| Controls | 1.84 ± 0.61 (4) |
| Phenylhydrazine | 3.77 ± 0.51* (6) |

The levels of 8-oxodGuo were expressed as the ratio of 8-oxodGuo/$10^5$ 2′-deoxyguanosine (dGuo). Phenylhydrazine treatment was performed as reported in Table 1. Results are means ± SEM. The number of animals is reported in brackets.
* Significantly different from control, $P < 0.05$.

hand, in one case (rat no. 3), the increase in free iron was not so pronounced, and no DNA fragmentation was observed (Fig. 3).

It is well known that hydroxyl radical produced by metal catalysis via the Fenton reaction generates a multiplicity of products from all four DNA bases, and that this pattern appears to be a diagnostic "finger-print" of ·OH attack [46]. The most commonly produced altered base, and the one most often measured as an index of oxidative DNA damage, is 8-oxo-7,8-dihydro-2′-deoxyguanine (also known as 8-hydroxyguanine). This is often measured as the nucleoside, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo). As shown in Table 4, the level of 8-oxodGuo in liver DNA from rats treated with phenylhydrazine for 6 days (usual protocol) was markedly increased as compared to untreated controls.

We investigated whether a persisting insult to cellular DNA might result in an alteration of gene expression, as assessed by the appearance of phenotypic changes. Table 5 shows that the administration of phenylhydrazine twice a week over a period of 1–3 weeks resulted in a progressive increase in both liver free iron level and liver DNA fragmentation. Fig. 4 shows that the same treatment also resulted, at the end of the sixth week, in a remarkable increase in the expression of γ-GT in hepatocytes. γ-GT-positive cells appeared in small *foci*, in regions of hepatic parenchyma far removed from periportal spaces (where γ-GT positive cells are also occasionally detectable under normal conditions). No signs of liver injury, particularly necrosis, were evident in serial sections for routine histology. Furthermore, no increase in serum lactate dehydrogenase was found in the same animals.

**TABLE 5. Liver free iron and DNA fragmentation in rats treated with phenylhydrazine twice a week for 1–3 weeks**

| | Liver free iron nmol/gr | DNA fragmentation |
|---|---|---|
| Controls | 4.2 | − |
| Phenylhydrazine 1 week | 26.4 | − |
| Phenylhydrazine 2 weeks | 64.8 | +− |
| Phenylhydrazine 3 weeks | 77.6 | ++ |

Phenylhydrazine was given s.c. at the dose of 0.1 mmol/kg body wt. on the first and third day of the week. The rats were sacrificed at the end of the first, second and third week.

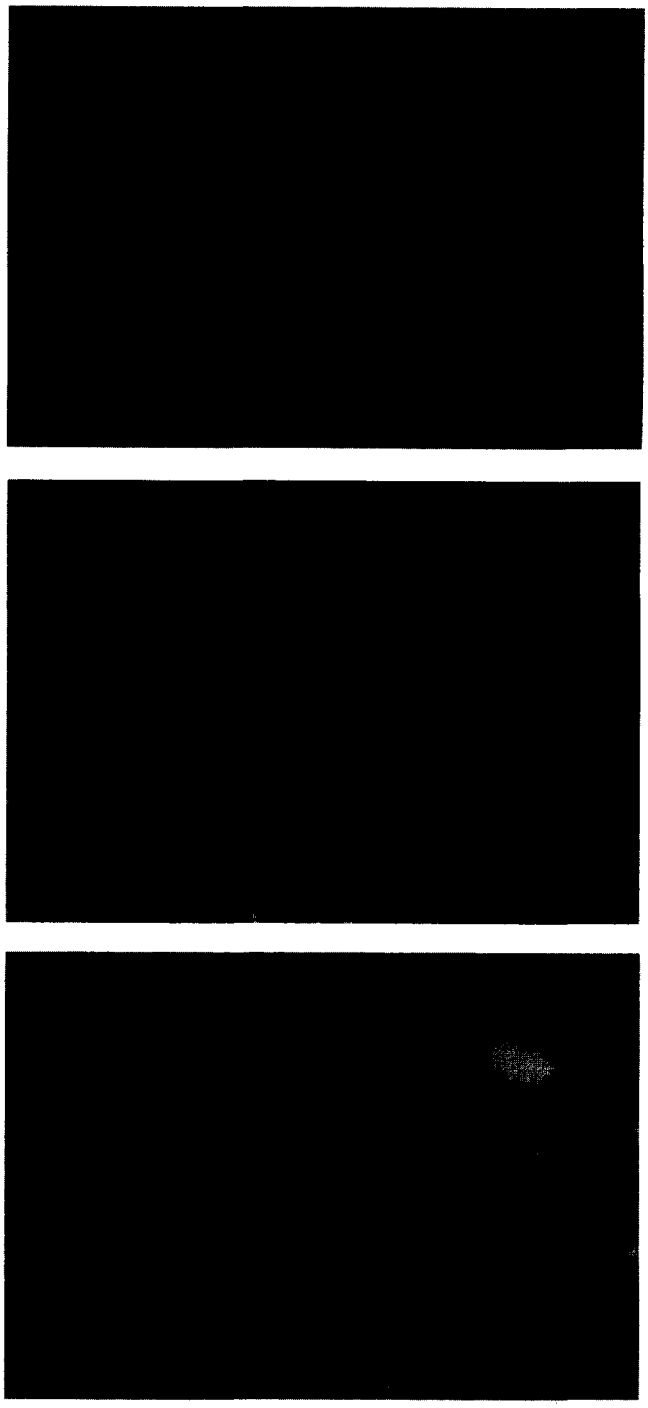

**FIG. 4. Focal re-expression of γ-glutamyl transpeptidase activity in hepatocytes of rats treated chronically (six weeks) with phenylhydrazine. Acetone-fixed cryostat sections (15 μm; see Materials and Methods). ×50. A, B, Phz-treated; γ-GT positivity involving single or small groups of hepatocytes is evident. C, control liver; γ-GT positivity is restricted to the biliary epithelium in portal spaces.**

## DISCUSSION

The present results show that the repeated treatment of rats with the hemolytic agent phenylhydrazine results in a massive influx of iron to the liver, with a marked increase in hepatic free, redox-active iron. As in secondary

hemocromatosis, iron found in the liver derives from hemoglobin, following massive hemocatheresis. The liver content of ferritin is also increased, as is the degree of its saturation by iron. An increase in free iron levels was also reported during the hepatic iron overload obtained by means of an iron-enriched diet (carbonyl iron, 2.5%) [47]. However, in the latter case a more than 4-fold increase in total iron (at 20 days of feeding) was accompanied by only a minor increase (1.3-fold) in free iron, and a 10-fold increase in total iron (at 40 days) was matched by only a 3-fold increase in free iron. Interestingly, in the phenylhydrazine-induced hepatic iron overload, the increase (7-fold) in free iron was on the contrary much higher than that of total iron (<2-fold). It is therefore conceivable that with a gradual uptake of iron, such as during an iron-enriched diet, the ability of the liver cell to sequester iron (possibly by increasing production of ferritin) is maintained, and the level of free iron is kept relatively low. Conversely, when a massive uptake of iron suddenly occurs, as in the case of the extensive hemocatheresis produced by phenylhydrazine, the capacity of the hepatocyte to keep iron complexed in storage forms probably becomes overwhelmed, with a subsequent increase in low molecular weight, catalytically active iron. Indeed, data reported in Table 2 show that a moiety of erythrocyte iron arriving at the liver is detectable in the protein-free ultrafiltrate, and is therefore interpretable as free iron. As far as the question, as to which hepatic cells free iron is located in, the fact that an increase in iron radioactivity was found in the soluble fraction (35880 vs 13360 cpm/g equivalent in controls) makes it likely that such an increase is attributable to the hepatocellular component of the liver (Kupffer cells and other non-parenchymal cells are likely sedimented during the centrifugations of the homogenate). This was further confirmed by the fact that—as mentioned above (see Results)—increased free iron was found in hepatocytes isolated from the livers of phenylhydrazine-treated rats.

Thus, it seems that phenylhydrazine intoxication establishes a model of hepatic iron overload particularly suitable for the study of the pathological events possibly elicited by the elevated free iron levels in the liver cell. In fact, the most interesting feature of this model appears to lie in the fact that—in face of a limited increase in total liver iron—a remarkable increase is induced in the free iron pool. One of the events induced by elevated free iron levels is the damage to hepatocellular DNA. Such damage consists of DNA fragmentation or strand breakages likely occurring at random, and is apparently related to the hepatic concentration of free iron. Indeed, in the experiments carried out with various dosages of phenylhydrazine (Table 4), as well as in additional experiments with carbonyl iron given for 3 to 6 weeks (not shown), DNA fragmentation seems to occur only when the hepatic concentration of free iron exceeds critical values (approx. 30–40 nmol/g). Critical values of total iron (non-heme iron) were also reported [13] for the onset of lipid peroxidation in liver mitochondria and microsomes of rats fed carbonyl iron for several weeks. These values (1,000–4,000 μg/g liver) were much higher than those (153.5 μg/g) obtained in the present phenylhydrazine-induced hepatic iron overload. In the latter, no signs of *in vivo* lipid peroxidation were found, and the observed increase in TBARS formation upon incubation of liver preparations from phenylhydrazine-treated rats only points to an increased susceptibility to lipid peroxidation, allegedly due to the increased content of free, redox-active iron.

In principle, DNA damage could also be due to phenylhydrazine itself (for instance, formation of reactive intermediates from the hepatic metabolism of phenylhydrazine). This possibility was actually ruled out by experiments with isolated hepatocytes (Fig. 2), in which the exposure to phenylhydrazine did not induce any DNA fragmentation. On the other hand, a likely mechanism for the observed iron-dependent DNA damage could be the action of reactive oxygen species generated by redox cycling iron in the Fenton reaction. This possibility is supported by the finding of an increased level of 8-oxodGuo in liver DNA of phenylhydrazine-treated animals. As mentioned above, the formation of 8-oxodGuo is a clear indication of ·OH attack; the occurrence of such oxidative damage means that ·OH is generated in near proximity of DNA. The metal catalysis necessary for its generation is likely accomplished by free iron, possibly bound to DNA itself. An ATP-dependent iron transport system has been recently described in isolated liver nuclei [48]. Iron is taken up by the nuclei from Fe-citrate, i.e., a small chelate in which iron can be in a redox-cycling form. It may be speculated that under conditions in which free iron levels are increased in the cytoplasm, as in iron overload, the transport is increased and a larger pool of free, redox-active iron is available in the nucleus, and is liable to cause oxidative damage to DNA.

In a number of *in vitro* studies [27–30], iron was reacted with isolated DNA and various DNA alterations were observed. $Fe^{2+}$ ions can be coordinated with ring nitrogens of DNA bases and perhaps also with a phosphate group in the same DNA strand [49]. A few studies have also been carried out *in vivo;* in one [50], the administration of Fe-nitrilotriacetate to rats caused modifications of all DNA bases in renal chromatin. Pyrimidine- and purine-modified molecules are typical products of · OH reactions with DNA bases (8-oxo-7,8-dihydro-guanine and 8-oxo-7,8-dihydro-adenine in particular) [51]. In another study [52], an increased unwinding of hepatic double-strand DNA was reported in rats with chronic dietary iron overload.

Conditions of chronic iron overload have been reported to be associated with increased occurrence of neoplasia, as in the case of liver tumor in human hemochromatosis [11] and colon cancer in human and in mice with high dietary iron intake [53–55]. The oxidative phenomena and the DNA alterations detectable during the induction of cancer in rat kidney by the administration of the known nefrocarcinogen, ferric nitrilotriacetate, have been studied [50, 56].

In our experimental model, prolonged exposure to increased free iron levels following treatment with phenylhy-

drazine resulted in the appearance of focal reexpression of γ-GT activity by hepatocytes. An increase in γ-GT activity and expression has recently been reported in hepatic overload induced by dietary administration of carbonyl iron [57]; due to the central role played by this enzyme in the cellular uptake of precursor aminoacids for the synthesis of the major antioxidant peptide glutathione [58], the finding has been interpreted as an adaptive response of the hepatocytes to the oxidative stress caused by iron overload [57]. On the other hand, γ-GT reexpression is also observed under a number of experimental conditions of liver tumor induction by chemicals. In selected experimental models, the focal reexpression of γ-GT activity by hepatocytes has been shown in fact to be among the first detectable signs of phenotypic alteration in chemically transformed hepatocytes [59, 60]; therefore, γ-GT reexpression in hepatocytes has been considered to be closely associated with multistage hepatocarcinogenesis [61]. It is tempting to speculate that under the experimental conditions reported in the present study as well, the reexpression of γ-GT represents the marker of a commitment of hepatocytes toward neoplasia, as is the case in a number of other known experimental situations. It seems unlikely that γ-GT reexpression is the result of a direct interaction of phenylhydrazine with DNA: in fact, as shown above, phenylhydrazine did not produce any DNA damage *in vitro* in isolated hepatocytes.

In conclusion, the sequence of events envisaged as the result of this work could be the following: phenylhydrazine-induced iron release in the erythrocyte → formation of senescent cell antigen → hemocatheresis → (6 days) → marked increase of hepatic free iron → DNA oxidative damage → (6 weeks) → appearance of γ-GT-positive foci → (??) further evolvement of the lesions.

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