Role of Lipid Peroxidation in Dapsone-Induced Hemolytic Anemia

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ABSTRACT

Dapsone hydroxylamine (DDS-NOH) is a direct-acting hemolytic agent responsible for dapsone-induced hemolytic anemia in the rat. The hemolytic activity of DDS-NOH is associated with the formation of disulfide-linked hemoglobin adducts on membrane skeletal proteins. We have postulated that this membrane protein "damage" is a consequence of DDS-NOH-induced oxidative stress within the red cell and that it serves as the trigger for premature removal of injured but intact red cells from the circulation by splenic macrophages. Oxidative stress has also been associated with the induction of lipid peroxidation, and it is possible that direct damage to the lipoidal membrane may play a role in the premature sequestration of the damaged cells in the spleen. To investigate this possibility, rat and human red cells were incubated with hemolytic concentrations of DDS-NOH and examined for evidence of lipid peroxidation using two independent assays: thiobarbituric acid-reactive substances formation and cis-paranarcic acid degradation. Phenylhydrazine, which is known to induce lipid peroxidation in red cells, was used as a positive control. The extent of thiobarbituric acid-reactive substances formation and cis-paranarcic acid degradation in DDS-NOH-treated rat and human red cells was not significantly different from that in control cells. In contrast, thiobarbituric acid-reactive substances formation and cis-paranarcic acid degradation were significantly increased in red cells treated with hemolytic concentrations of the positive control, phenylhydrazine. These data suggest that lipid peroxidation is not involved in the mechanism underlying dapsone-induced hemolytic anemia.

The arylamine drug dapsone is used for the treatment of a number of skin disorders, including dermatitis herpetiformis and Hansen disease (Thuong-Nguyen et al., 1993), and is an important component in the treatment and prevention of opportunistic infections, such as Pneumocystis carinii pneumonia and toxoplastic encephalitis, in AIDS patients (Lee et al., 1989; Torres et al., 1993). The effectiveness of dapsone therapy, however, is frequently limited by its capacity to induce hemolytic anemia and methemoglobinemia. The hemotoxicity of dapsone is mediated by its N-hydroxy metabolites, DDS-NOH and monoacetyl DDS-NOH (Grossman and Jollow, 1988). These metabolites are direct-acting and equipotent hemolytic agents in red cells isolated from rats, and they are formed in vivo after administration of dapsone in amounts sufficient to account for the hemolytic activity of the parent drug. Thus, when rat 51Cr-labeled red cells are exposed to DDS-NOH in vitro and then returned to the circulation, the labeled cells are removed rapidly from the circulation by the spleen. This experimental model has allowed us to examine the potential relevance of cellular changes provoked in red cells exposed to hemolytic concentrations of DDS-NOH in vitro to induction of premature splenic sequestration in vivo.

The hemolytic activity of arylamine drugs in humans has long been associated with a drug-induced oxidative stress within the red cell, as evidenced by depletion of cellular GSH and by the enhanced sensitivity to hemolytic anemia seen in red cells deficient in erythrocytic glucose-6-phosphate dehydrogenase activity (Beutler, 1969; Grossman et al., 1995).

Oxidative stress within red cells is thought to result from a cyclic oxidation-reduction reaction that occurs between the arylhydroxylamine metabolite and oxyhemoglobin, yielding the nitrosoarene and methemoglobin, respectively (Kiese, 1974). A number of active oxygen species and sulfur free radicals are produced as a consequence of this cyclic redox reaction (Rostorfer and Cormier, 1957; Ellederová et al., 1968; Kiese, 1974; Maples et al., 1990; Bradshaw et al., 1995), and one or more of these species may be responsible for the damage that initiates removal of the injured cell from the circulation.

In studies on the effects of in vitro exposure to DDS-NOH...
in rat and human red cells, we have observed a rapid loss of GSH with concomitant formation of glutathione-protein mixed disulfides, disulfide-linked hemoglobin polymers and disulfide-linked hemoglobin adducts on certain membrane skeletal proteins (Grossman et al., 1992; McMillan et al., 1995). Furthermore, we have reported recently that hydroxyl radical and ferrylhemoglobin are generated in rat red cells exposed to hemolytic concentrations of DDS-NOH (Bradhshaw et al., 1997). These data have led to the hypothesis that oxygen-centered free radicals induce formation of both glutathione and hemoglobin thyl radicals and that the latter react with skeletal proteins of the cell membrane to form hemoglobin-skeletal protein adducts. This “damage” to critical membrane skeletal proteins is thought to trigger premature sequestration of the injured red cells by the spleen (Jollow et al., 1995).

An alternative hypothesis is that splenic sequestration of DDS-NOH-treated red cells is the result of damage to membrane lipids rather than (or in addition to) damage to membrane skeletal proteins. Peroxidation of membrane lipids has been implicated in cellular injury induced by a variety of toxicants that either have sufficient reactivity to initiate a free radical chain reaction (i.e., a compound-centered free radical) or can generate reactive oxygen free radicals within cells (for review, see Horton and Fairhurst, 1987; Comporti, 1993). Hemolytic arylamines have long been accepted as being capable of generating active oxygen and other free radical species, and it is on this basis that lipid peroxidation has been proposed to be involved in the hemolytic response induced by these compounds. However, the ability of hemolytic arylhydroxylamine metabolites to initiate red cell lipid peroxidation has yet to be demonstrated experimentally.

The present studies were undertaken to determine whether erythrocyte membrane lipid peroxidation could be detected in rat red cells incubated in vitro with hemolytic concentrations of DDS-NOH, and thus whether lipid peroxidation could have a role in dapsone-induced hemolytic anemia. Lipid peroxidation was assessed using the TBARS assay and by monitoring the oxidative degradation of the fluorescent fatty acid probe cPnA incorporated into erythrocyte membranes. Furthermore, the classical hemolytic agent phenylhydrazine, which is well known to induce lipid peroxidation in red cells (Goldberg and Stern, 1977; Clemens et al., 1984), was used as a positive control in these studies. We report that no evidence of red cell membrane lipid peroxidation was detected in rat red cells exposed to hemolytic concentrations of DDS-NOH. In addition, lipid peroxidation could not be detected in parallel studies with DDS-NOH-treated human red cells. Thus these data do not support the hypothesis that lipid peroxidation is responsible for the cellular damage induced by hemolytic arylamine compounds.

**Materials and Methods**

**Chemicals and materials.** DDS-NOH was synthesized as described previously (Grossman and Jollow, 1988). Phenylhydrazine and MDA were purchased from Sigma Chemical Co. (St. Louis, MO). cPnA was purchased from Pierce Chemical Co. (Rockford, IL). All other chemicals and reagents were of the best commercially available grade.

**Animals.** Male Sprague-Dawley rats (130–150 g) were purchased from Charles River (Raleigh, NC) and maintained on food and water ad libitum. Animals were acclimated for 1 week to a 12-hr light-dark cycle before their use. Red blood cells from anesthetized rats and red cells from human volunteers were collected into heparinized tubes and washed three times with isotonic phosphate-buffered saline supplemented with 10 mM d-glucose (PBSG, pH 7.4) to remove the plasma anduffy coat. The cells were resuspended in PBSG and used the same day they were collected.

**Determination of the hemolytic response.** The survival of rat 

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in vivo

was determined as described previously (Harrison and Jollow, 1986) after in vitro exposure of the labeled cells to DDS-NOH or phenylhydrazine. DDS-NOH (in 10 μl of acetone) or phenylhydrazine (in 30 μl of PBSG) was added to red cell suspensions (40% hematocrit, 3.5 ml) and allowed to incubate aerobically at 37°C. After 2 hr of incubation, the cells were washed once and resuspended in PBSG. Aliquots of the suspensions (0.5 ml) were administered i.v. to isologous rats, and a T0 sample was taken from the orbital sinus 30 min after administration of the labeled cells. Additional blood samples were taken at 48-hr intervals for 14 days. At the end of the experiment, the radioactivity in the blood samples was counted in a well-type gamma counter, and the counts above background were expressed as a percentage of the T0 sample. The time for blood radioactivity to decrease to 50% of initial levels (T50) was determined for each animal by regression analysis. Statistical significance was determined with the use of Student’s t test.

**Determination of TBARS in red cells.** TBARS formation was measured as described previously (Placer et al., 1966). Briefly, erythrocyte suspensions (40% hematocrit in 2.5 ml of PBSG) were incubated with DDS-NOH or phenylhydrazine at 37°C. After incubation, aliquots of the cells (0.75 ml) were lysed with 375 μl of 30% TCA and centrifuged (2 min; 13,000 × g). The supernatants (0.75 ml) were incubated 1:1 (v/v) with thiobarbituric acid (0.67%) at 100°C for 15 min. The samples were allowed to cool to room temperature, and absorbance was read at 532 nm. TBARS formation was quantitated with a standard curve using known amounts of MDA.

**Determination of cPnA degradation in red cells.** cPnA degradation was monitored continuously within intact red cells as described by van den Berg et al. (1991). Incorporation and degradation of cPnA in erythrocyte membranes were determined at excitation and emission wavelengths of 312 and 455 nm, respectively, in an SLM Aminco Instrumente 8000 C spectrofluorometer (excitation slit width, 4 nm; emission slit, 16 nm) interfaced to an IBM 486 computer. Experiments were initiated by placing cPnA-labeled erythrocytes (diluted to a 1% cell suspension) in a thermostated cuvette (total volume, 2.5 ml) that was continuously stirred within the spectrofluorometer at 25°C. After a 2-min preincubation, DDS-NOH, phenylhydrazine or vehicle (acetone or PBSG, 2.5 μl) was injected through a septum into the cuvette, and the fluorescence signal was recorded continuously for up to 15 min.

In a separate series of experiments, the cPnA fluorescence signal intensity was measured in hemoglobin-free membrane ghosts (unsealed membrane vesicles) prepared from cPnA-labeled red cells exposed to DDS-NOH or phenylhydrazine. DDS-NOH or phenylhydrazine was added to red cell suspensions (40% hematocrit, 2.5 ml) and incubated aerobically for 1 hr at 37°C. After the incubation period, aliquots of the cells (0.4 ml) were removed and lysed in the dark in 8.0 ml of ice-cold, argon-purged lysis buffer (5 mM KH2PO4, pH 8.0) containing an inhibitor of proteolysis (phenylmethyl sulfonfylfluoride, 100 μM) and an antioxidant (BHT, 50 μM). The ghosts were pelleted by centrifugation (20,000 × g, 15 min) and washed three times in argonated lysis buffer (1.0 ml) containing 5 mM DTT until the membrane-bound hemoglobin was removed as judged by the bleached color of the protein. Ghosts were also prepared from untreated red cells immediately after incorporation of cPnA (T0 sample).

After the final wash, aliquots of the pelleted ghost proteins were saved for determination of protein concentration (Bio-Rad); the remaining protein samples were maintained in the dark on ice under argon to minimize degradation of the fluorescent probe while awaiting analysis. Aliquots of the protein samples were injected through a
septum into a cuvette containing phosphate buffer (final volume, 2.5 ml), which was continuously stirred within the spectrofluorometer. The fluorescence signal intensity was recorded at excitation and emission wavelengths of 318 and 410 nm, respectively, against an identical sample blank that did not contain cPnA. Percent cPnA degradation that occurred during the 1-hr incubation period was calculated as a percentage of signal intensity recorded in the $T_0$ sample.

Results

Hemolytic activity of phenylhydrazine vs. DDS-NOH in rat red cells. Phenylhydrazine was employed as a positive control in these studies because it has long been known to induce a hemolytic response in experimental animals and because its ability to induce lipid peroxidation in red cells is well accepted (Jain and Hochstein, 1979; Goldstein et al., 1980). However, the hemolytic activity of phenylhydrazine under the experimental conditions used for DDS-NOH (viz., incubation of rat $^{51}$Cr-labeled red cells in vitro for 2 hr, followed by measurement of survival in vivo after administration of the labeled cells to the circulation of isologous rats) has not been reported. Because the objective of these studies was to compare the extent of lipid peroxidation under incubation conditions that result in a similar degree of hemolytic activity, initial studies were performed to characterize the hemolytic activity of phenylhydrazine according to the DDS-NOH experimental protocol.

In agreement with previous studies (Grossman and Jollow, 1988), exposure of the radiolabeled red cells to DDS-NOH resulted in a dramatic increase in the rate of removal of radioactivity from the circulation of rats as compared with controls (fig. 1A). The hemolytic response induced by DDS-NOH, determined as the percent reduction in the time required for removal of 50% of the blood radioactivity ($T_{50}$), was concentration-dependent, with an $EC_{50}$ of about 130 $\mu$M (fig. 2). Incubation of the radiolabeled red cells with phenylhydrazine also induced an increase in the rate of removal of the cells from the circulation (fig. 1B). The hemolytic response induced by phenylhydrazine was also concentration-dependent, with an $EC_{50}$ of about 800 $\mu$M and a maximally effective concentration of about 5 mM (fig. 2). Notably, the curves for blood radioactivity vs. time course for phenylhydrazine and DDS-NOH (fig. 1, A and B) were similar in shape. Both compounds were equally efficacious; however, DDS-NOH was about 6-fold more potent than phenylhydrazine (fig. 2).

To determine whether significant lysis of the red cells had occurred during the 2-hr incubation period with DDS-NOH and phenylhydrazine (i.e., before readministration of the cells to isologous rats), the amount of hemoglobin and radioactivity released into the supernatant was measured after centrifugation of the cells. The extent of hemolysis that occurred during the 2-hr incubation period in red cell suspensions treated with either DDS-NOH (80–200 $\mu$M) or phenylhydrazine (0.2–5 mM) was less than 1% and was not significantly different from untreated controls.

Formation of TBARS in DDS-NOH-treated red cells. To determine the extent of lipid peroxidation, TBARS...
formation was assessed after *in vitro* incubation with DDS-NOH and phenylhydrazine across a range of concentrations that induced a hemolytic response. As shown in figure 3, the amount of TBARS formed in phenylhydrazine-treated red cells during a 60-min incubation was markedly increased as compared with that of the control. The increase in TBARS formation was dependent on phenylhydrazine concentration, and it occurred at all concentrations of phenylhydrazine that provoked a hemolytic response. In contrast, incubation of the cells with DDS-NOH caused no increase in TBARS at any concentration (fig. 3).

Upon further examination of the data in figure 3, DDS-NOH appeared to induce a slight concentration-dependent decrease in TBARS formation, which raised the possibility that DDS-NOH might interfere with the spectrophotometric assay. To examine this possibility, DDS-NOH was added to blood containing known amounts of MDA, and the samples were analyzed immediately. The data indicated that 180 μM DDS-NOH produced about a 15% reduction in absorbance of MDA at 532 nm. When the experimental data in figure 3 were corrected for this interference, there remained no significant difference between DDS-NOH-treated red cells and controls. Furthermore, when the cells were allowed to incubate for 60 min in the presence of both DDS-NOH and MDA, the magnitude of the interference was the same, which indicated that DDS-NOH did not accelerate MDA degradation.

**cPnA degradation in DDS-NOH-treated red cells.** Although we observed no evidence for DDS-NOH-induced lipid peroxidation when TBARS was used as an indicator, problems with the reliability of this assay are well known (Janero, 1990). Thus it was necessary to confirm the results of the TBARS assay using another indicator of lipid peroxidation. Measurement of the oxidative degradation of the fluorescent fatty acid probe cPnA has been used as a direct and continuous assay of lipid peroxidation (Kuypers et al., 1987). This method is considered ideal because cPnA is fluorescent only when incorporated into membranes, and it reacts with a variety of free radicals to yield nonfluorescent products. The cPnA degradation assay has been used to measure lipid peroxidation *in situ* in liposomal membranes and red cell membrane ghosts (van den Berg et al., 1988; McKenna et al., 1991; Hedley and Chow, 1992) and has also been adapted for use with intact red cells (van den Berg et al., 1991). However, because of quenching of the fluorescence signal by hemoglobin and by the light-scattering effects of the red cells, the assay requires ≤1% cell concentration in suspensions and constant stirring to prevent sedimentation of the cells during analysis.

For the present experimental purpose, fluorescence intensity was monitored in cPnA-labeled red cell suspensions at a low hematocrit (1% cell concentration) treated with low concentrations of DDS-NOH (about 3–15 μM) or phenylhydrazine (about 20–100 μM). These relatively low concentrations were utilized to maintain the same ratio of hemotox- icant to red cell number as that used in the 51Cr hemolytic assay. When cPnA-labeled red cells were placed in the spectrophotometer, a fluorescence signal was recorded that degraded slowly; less than ~5% decrease occurred over a 15-min time period. The rate of decay did not change when the vehicle (2.5 μl of acetone or PBSG) was added through the septum into the cuvette (fig. 4). However, when phenylhydrazine (50 μM) was added to the cuvette (fig. 4), there was a slight, transient increase in signal intensity, followed by an increase in the rate of fluorescence signal decay, as compared with the control, which continued throughout the incubation period. In contrast, no increase in the rate of fluorescence decay was observed when DDS-NOH (15 μM) was injected into the cuvette (fig. 4). In fact, no increase in the rate of fluorescence decay could be observed in DDS-NOH-treated red cells, even when the concentration of DDS-NOH was increased to levels as high as 150 μM (data not shown).

**cPnA degradation under hemolytic conditions.** To determine the extent of lipid peroxidation under hemolytic conditions, cPnA-labeled red cells were suspended at a normal hematocrit (i.e., 40% cell concentration) and exposed to hemotoxic concentrations of DDS-NOH or phenylhydrazine. After incubation for 1 hr at 37°C, the cells were lysed and membrane ghosts were prepared. Red cell ghost preparation was conducted in the dark under anaerobic conditions to minimize cPnA degradation during sample preparation and analysis. The amount of cPnA degradation during the 1-hr incubation period is expressed as a percentage of the fluorescence signal in control ghosts that did not undergo incubation (T₀ sample). As shown in figure 5, ghosts from untreated control cells exhibited about a 20% loss in signal intensity after a 1-hr incubation compared with that of ghosts prepared immediately after cPnA incorporation.

In preliminary studies, ghosts prepared from red cells treated for 1 hr with both DDS-NOH and phenylhydrazine showed significant signal decay (>50%) as compared with untreated cPnA-labeled control cells. However, both DDS-NOH- and phenylhydrazine-treated cells contained clearly visible membrane-bound hemoglobin, which is known to quench cPnA fluorescence strongly (van den Berg et al., 1988). The hemoglobin in DDS-NOH- and phenylhydrazine-treated red cell ghosts is linked to membrane skeletal protein through a mixed disulfide (Vilsen and Nielsen, 1984; Grossman et al., 1992) and can be removed by treatment of the ghosts with the disulfide-reducing agent DTT. When the experiment was repeated using DTT-treated ghosts, significant fluorescence decay could still be observed in phenylhydrazine-treated red cells (fig. 5). In contrast, no significant

![Fig. 3. Formation of TBARS in rat erythrocytes exposed *in vitro* to DDS-NOH and phenylhydrazine. Rat erythrocytes (40% hematocrit) were incubated in PBSG containing the indicated concentrations of DDS-NOH and phenylhydrazine for 60 min at 37°C. Control cells were incubated with the vehicle alone. After incubation the erythrocytes were lysed and centrifuged, and the supernatant was analyzed for TBARS as described in "Materials and Methods." The values are means ± S.D. (*P < .05, n = 3).](image)
increase in cPnA degradation was observed in DTT-treated ghosts from red cells incubated with DDS-NOH. These data indicated that the modified cPnA assay could be used to monitor lipid peroxidation under hemolytic conditions and confirmed that lipid peroxidation was not detectable in rat red cells exposed to DDS-NOH.

Lipid peroxidation in human erythrocytes. To determine whether the responses observed in rat red cells regarding lipid peroxidation are applicable to humans, we examined TBARS formation and cPnA degradation in human red cell suspensions exposed to DDS-NOH and phenylhydrazine. Previous studies comparing the sensitivity of rat vs. human red cells to oxidative stress indicated that human red cells may be about 2- to 3-fold less sensitive to DDS-NOH-induced hemolytic injury than are rat red cells. The EC_{50} for the hemolytic response in human red cells has been estimated to be in the range of 300 to 350 μM DDS-NOH (McMillan et al., 1995). Thus human red cell studies were conducted using higher concentrations of DDS-NOH (175–500 μM).

As shown in figure 6, the amount of TBARS formed in human red cells exposed to phenylhydrazine was significantly increased compared with controls. In contrast, no increase in TBARS was observed in human red cells at any concentration of DDS-NOH (fig. 6).

cPnA studies in human red cells were conducted under the low hematocrit conditions (1% cell concentration) with correspondingly lower concentrations of the hemotoxictants. As shown in figure 7A, addition of phenylhydrazine to cPnA-labeled human red cells induced an increase in the rate of fluorescence signal decay as compared with the vehicle control. The response appeared to be concentration-dependent; however, in contrast to the rat red cells, the decrease in
signal intensity in human cells lasted for only about 3 min after the addition of phenylhydrazine. No increase in the rate of signal decay was observed in human red cells treated with 50 μM DDS-NOH (fig. 7B).

Discussion

Lipid peroxidation has long been considered to play a role in the mechanism underlying arylamine drug-induced hemolytic anemia. Much of the basis for this concept comes from early studies, such as those by Rasbridge and Scott (1973), who showed that red cells from dapsone patients were unusually sensitive to peroxide-induced lysis. Other investigators have demonstrated the correlation between hydrogen peroxide formation in red cells treated with various hemolytic agents, and lipid peroxidation and hemolysis (Cohen and Hochstein, 1964; Stocks and Dormandy, 1971; Ferrali et al., 1992). Goldstein and McDonagh (1976) reported that although there was no evidence for an increase in blood levels of MDA in dapsone-treated rats, spectra consistent with a fluorescent cross-link of MDA with aminolipid were observed. However, as noted by the authors, the presence of membrane-bound hemoglobin complicated interpretation of the results; heme is a potentiator of lipid peroxidation and could have produced artifactual peroxidation of membrane lipids. Thus a role for lipid peroxidation in arylamine-induced hemolytic anemia has not been firmly established. Furthermore, many of the early studies described above utilized the parent arylamine as the test compound in isolated red cell incubations before it was appreciated that metabolite(s) are responsible for the toxicity.

The concept of lipid peroxidation as the triggering mechanism for cell lysis is not unreasonable, given the fact that the red cell is particularly susceptible to peroxidation of lipids because of the relatively high content of polyunsaturated fatty acids in the membrane and the presence of very high concentrations of iron (in the form of hemoglobin). Although iron bound within hemoglobin is considered not to be capable of supporting a Fenton-type reaction sequence to generate the highly reactive hydroxyl radical (Gutteridge, 1986; Puppo and Halliwell, 1988), iron has been shown to be released in a diffusible form as a consequence of heme oxidation in red cells exposed to a variety of oxidizing agents (Ferrali et al., 1992; Ciccoli et al., 1994). Free or “diffusible” iron is then available to react with hydrogen peroxide to form hydroxyl radical, which is a well-known initiator of lipid peroxidation. Highly reactive ferryl heme species, formed by the reaction of hydrogen peroxide with hemoglobin, may also have the capacity to damage membrane lipids (Walters et al., 1983; Galairis et al., 1990). The consequence of membrane lipid peroxidation to the red cell is distortion of the structure of the lipid bilayer, resulting in loss of membrane fluidity, inactivation of enzymes, electrolyte leakage and a concomitant influx of water into the cell, leading to cellular swelling and lysis (Weed and Reed, 1966; Horton and Fairhurst, 1987).

We have provided evidence that hemolytic damage caused by dapsone and its metabolites is associated with protein oxidation (Jollow et al., 1995). The present studies were undertaken to examine the alternative hypothesis: that peroxidation of lipid is a key event in dapsone-induced hemolytic anemia. Two independent assays for lipid peroxidation were performed in rat and human erythrocyte suspensions treated with hemolytic concentrations of DDS-NOH. Under the experimental conditions that result in splenic sequestration of damaged red cells when they are readministered to rats, DDS-NOH-induced lipid peroxidation was not detectable. In contrast, equihemolytic concentrations of the positive control, phenylhydrazine, caused lipid peroxidation in rat and human red cells, as evidenced by increases in the formation of TBARS and in the rate of cPnA degradation.

TBARS is the most frequently utilized assay for lipid peroxidation in the literature. However, it is well appreciated that false negatives occur, and hence results showing no increase in TBARS must be interpreted carefully (Janero, 1990). In the present studies, we examined TBARS formation in DDS-NOH-treated red cell incubates under conditions that are relevant to the in vivo toxic response, and we utilized phenylhydrazine as a positive control for the induction of lipid peroxidation in red cells. Under these conditions, the lack of an increase in TBARS formation in red cell incubates containing DDS-NOH can be interpreted as a lack of formation rather than an inability to detect TBARS. It is interesting to note that DDS-NOH did have an effect on our ability to
measure TBARS formation in red cells, as evidenced by the diminution of the fluorescence signal of exogenously added MDA; however, the quenching effect was minimal and would not have been sufficient to mask an increase in TBARS formation if one had occurred.

The results of the TBARS assay were confirmed by the cPnA degradation assay. In contrast to TBARS, which are unstable end products of lipid peroxidation, oxidation of membranes in intact red cells loaded with cPnA can be followed continuously by monitoring the loss of cPnA fluorescence. This assay has been reported to work well in isolated membrane vesicles devoid of any spectrally interfering substances. In red cells, the scattering of light by the cells and the quenching of the fluorescence signal by hemoglobin and its oxidation products present major limitations (van den Berg et al., 1991). For the present experimental purpose, two types of studies using cPnA were performed: 1) reduced hematocrit (1% cell concentration), which permitted continuous monitoring but required marked reduction in the concentrations of DDS-NOH and phenylhydrazine that could be examined, and 2) normal hematocrit (40% cell concentration), which allowed the use of “hemolytic” concentrations of DDS-NOH and phenylhydrazine but restricted the number of observations that could be made.

The second type of study required significant modification of the standard cPnA protocol. Preliminary experiments established that cPnA-labeled red cells could be incubated for 1 hr at 37°C and then used for preparation of hemoglobin-free ghosts. Provided that the lysis and washing procedures were performed in the dark and under anaerobic conditions, loss of cPnA fluorescence was less than 20% as compared with that of ghosts prepared at T₀. An additional problem, however, became apparent. Red cells exposed to DDS-NOH and phenylhydrazine contained membrane-bound hemoglobin that caused extensive quenching of the fluorescence signal. This difficulty was overcome by the inclusion of the disulfide reducing agent DTT in the lysis/washing buffer in order to strip the disulfide-linked membrane-bound hemoglobin from the ghost protein (Grossman et al., 1992). Under these conditions, phenylhydrazine-treated red cells showed significant reduction in fluorescence intensity (fig. 5). In contrast, the extent of cPnA degradation in DDS-NOH-treated cells was not significantly different from that in the control red cells.

Although the objective of this study was to determine the role of lipid peroxidation in DDS-NOH-induced hemolytic anemia, some comments on phenylhydrazine-induced hemotoxicity are warranted. The ability of phenylhydrazine to initiate lipid peroxidation in red cells is well accepted. However, the mechanism underlying the hemolytic injury induced by phenylhydrazine, like that of the hemolytic arylamines, is not well defined. In particular, whether the lipid peroxidation observed in phenylhydrazine-treated red cells is causal in the hemolytic response has not been established. Hemoglobin oxidation, hemoglobin-skeletal protein adduct formation and skeletal protein damage (i.e., spectrin degradation) also occur and may play a role in the hemotoxicity of this compound (Vilsen and Nielsen, 1984; Arduini and Stern, 1985; Shetlar and Hill, 1985).

However, many of these studies have utilized relatively high concentrations of phenylhydrazine (e.g., 5–10 mM) in the red cell suspensions and have used lysis of the cells in vitro as the toxic endpoint. In the present studies, because phenylhydrazine was to be used as a positive control, it was necessary to establish the concentration range for phenylhydrazine-induced hemolytic anemia using the in vitro exposure/in vivo red cell survival assay system employed for DDS-NOH. Thus we examined the ability of phenylhydrazine to elicit an hemolytic response in vivo by measuring the survival of 51Cr-labeled rat red cells after in vitro exposure to the compound for 2 hr at 37°C. Phenylhydrazine-treated red cells were removed from the circulation in a concentration-dependent manner (fig. 1B) with an EC₅₀ (about 800 μM) notably lower than that observed when direct lysis is used as the toxic endpoint. Examination of the incubation media before readministration of the phenylhydrazine-treated cells for cell-free hemoglobin and cell-free 51Cr indicated that no significant lysis had occurred. These considerations raise the possibility that there may be two distinct hemolytic responses elicited by phenylhydrazine: one, at low concentrations, that causes premature splenic sequestration; and the other, at higher concentrations, that causes frank cellular lysis.

Currently, there is uncertainty in the literature with regard to the relative importance of lipid peroxidation vs. membrane skeletal protein damage in hemolytic injury (Winterbourn and Carrell, 1972; Vilsen and Nielsen, 1984) and the roles of intravascular lysis vs. splenic sequestration of intact red cells in the process by which damaged cells are removed. The present data strongly suggest that lipid peroxidation does not play a significant role in the premature removal of DDS-NOH-damaged red cells and hence that lipid peroxidation is not an obligatory component of the mechanism underlying the hemotoxicity of arylamine drugs and environmental chemicals.

The situation in regard to arylhydrazines, such as phenylhydrazine, is less clear. Extensive lipid peroxidation was evident after exposure to low concentrations (i.e., <1 mM) of phenylhydrazine (figs. 3 and 4). These concentrations also provoked premature removal of the cells from the circulation (fig. 1B) but did not cause cell lysis. The fate of these red cells in the body was not determined as part of this study; however, previous workers have suggested that cells with moderate damage are removed almost entirely by the spleen (Jandl and Tomlinson, 1958; Rothberg et al., 1959), whereas more severely damaged cells are removed by the reticuloendothelial system as a whole, the liver contributing most of the uptake capacity because of its large size and blood flow (Azen and Schilling, 1963; Azen and Schilling, 1964). Intravascular lysis has been reported but may be only a minor contributor to the removal of phenylhydrazine-damaged red cells (Azen and Schilling, 1964; Rifkind, 1965). In the present studies, the kinetics of removal of phenylhydrazine- and DDS-NOH-damaged cells were quite similar (fig. 1), which suggested that their mechanisms of toxicity are similar. DDS-NOH-damaged red cells are removed intact by the spleen (Grossman and Jollow, 1988) and not by intravascular lysis with subsequent removal of the cell fragments by both splenic and hepatic components of the reticuloendothelial system (Jandl et al., 1956). Additional studies are needed to establish the in vivo fate of rat red cells treated with these relatively low concentrations of phenylhydrazine.

The concept of two distinct mechanisms of red cell injury is supported by studies that have investigated the formation of free radical species in red cells exposed to arylhydroxy-
laminine prolylhydroxylase. In the case of hemolytic arylhydroxylamine metabolites, we have observed the formation of active oxygen species (hydroxyl radical and ferrylhemoglobin) and glutathione and hemoglobin thiol radicals (Bradshaw et al., 1995; Bradshaw et al., 1997). As illustrated in figure 8, we postulate that active oxygen species, formed as a consequence of arylhydroxylamine redox cycling with oxyhemoglobin, are too reactive to reach the lipid bilayer of the cell membrane and instead react with sulfur of GSH and hemoglobin, generating the corresponding thiol free radicals (Jollow et al., 1995). The thiol radicals have sufficient stability to reach the cell membrane and sufficient reactivity to interact with skeletal protein thiol groups, but they do not have sufficient lipid solubility to gain access to the lipid bilayer and initiate lipid peroxidation.

Phenyldiazine has also been shown to generate active oxygen species (Cohen and Hochstein, 1964; Misra and Fridovich, 1976) and thyl radicals (Maples et al., 1988), which may also be responsible for the damage observed in the skeletal protein of phenyldiazine-treated red cells. However, phenyldiazine has been shown to undergo oxidation to phenyl diazine and phenyl radical (Goldberg and Stern, 1977; Hill and Thornalley, 1981), which is considered to have sufficient lipophilicity to penetrate the lipid bilayer and hence initiate lipid peroxidation (fig. 8). Although nitrooxide free radicals have been observed by electron paramagnetic resonance spectroscopy in cell-free systems as intermediates in the oxidation of arylhydroxylamines (Maples et al., 1990), we found no evidence for their formation in red cells under hemolytic conditions (Bradshaw et al., 1995; Bradshaw et al., 1997), which may explain why no lipid peroxidation was observed in DDS-NOH-treated red cells.

In summary, the present data indicate that hemolytic concentrations of DDS-NOH do not induce peroxidation of membrane lipids in rat and human red cells. Thus lipid peroxidation appears to play no significant role in dapsone-induced hemolytic anemia. The situation with respect to phenyldiazine appears to be more complex; lipid peroxidation, but not direct lysis, occurs at the EC50 concentration (about 800 \( \mu \)M) as measured by the elimination of damaged \(^{51}\)Cr-labeled red cells from the circulation; however, direct lysis is known to occur at higher concentrations (5–10 mM). Thus we propose that there may be two distinct mechanisms of chemical-induced hemolytic injury that lead to the same toxic endpoint: 1) membrane protein damage with premature splenic sequestration and 2) membrane lipid peroxidation with subsequent direct lysis and/or sequestration by the reticuloendothelial system in general.

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Fig. 8. Postulated mechanisms for the induction of toxicity in red cells exposed to DDS-NOH and phenyldiazine. Both DDS-NOH and phenyldiazine induce the formation of active oxygen species, which are postulated to initiate cellular injury leading to splenic sequestration of red cells. Phenyldiazine may also operate via an alternative mechanism, leading to initiation of lipid peroxidation and vascular lysis. See the text for a detailed explanation. RES, reticuloendothelial system.

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