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Oxidative damage by phenylhydrazine diminishes erythrocyte anion transport

Howard R. Petty, Ming-jie Zhou and Zhili Zheng

Department of Biological Sciences, Wayne State University, Detroit, MI (U.S.A.)

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Human erythrocytes were exposed to oxidative stress by treatment with the slowly hemolytic drug phenylhydrazine. Phenylhydrazine has been previously shown to trigger the production of toxic oxygen metabolites including O_2^- and H_2O_2 and the formation of Heinz bodies. The concentration-dependent formation of Heinz bodies was confirmed using optical microscopy. Heinz body formation was accompanied by surface protuberances as shown by scanning electron microscopy. The formation of Heinz bodies was accompanied by inhibition of anion translocation. Anion translocation was measured using the anionic fluorescent substrate analog *N*-(2-aminoethylsulfonate)-7-nitrobenz-2-oxa-1,3-diazole (NBD-taurine). The efflux of NBD-taurine was measured by continuous monitoring of transport by fluorescence (CMTF). The mean value of the kinetic rate constant for transport, k, was found to be -0.090 ± 0.017 min^{-1}. Phenylhydrazine was found to decrease k to less than one-half of control values in a dose-dependent fashion. The disruption of anion translocation may be related to the oxidative effects of phenylhydrazine and to the generation of Heinz bodies, which bind to the N-terminal domain of band 3.

Introduction

Toxic oxygen radicals may participate in several forms of erythrocyte hemolysis. Stimulated immune effector cells such as neutrophils and macrophages produce large quantities of toxic oxygen radicals followed by the hemolysis of nearby or attached erythrocytes [1,2]. Certain congenital or acquired conditions can also lead to oxidant-mediated hemolysis. For example, toxic oxygen radicals have been implicated in β-thalassemia major, glucose-6-phosphate dehydrogenase deficiency, unstable hemoglobin diseases, erythrocyte aging, and other conditions [3–6]. Moreover, certain hemolytic drugs such as phenylhydrazine are known to produce toxic oxygen metabolites including superoxide anions and hydrogen peroxide [6,7].

Phenylhydrazine provides a convenient in vivo and in vitro model of oxidant- and Heinz body-associated hemolysis. Since phenylhydrazine-mediated hemolysis is relatively slow, it is amenable to physiological and biochemical analysis. Phenylhydrazine treatment alters cell deformability [8]. Several changes directly linked with the erythrocyte plasma membrane have also been observed. Heinz bodies attach to the inner surface of the erythrocyte's membrane via band 3 [9–11]. In addition to the polymerization of hemoglobin derivatives, the polymerization of membrane-associated proteins has also been observed [12]. Alterations in lipid packing, lipid peroxidation, lipid fluidity and phospholipid flip-flop have been reported [13–15]. Since colloid-osmotic forces may contribute to the hemolytic event, an understanding of membrane transport during oxidative stress is required. Early studies suggested that changes in internal sodium and potassium levels accompanied phenylhydrazine treatment [16]. Recently, Shalev et al. [17] have shown that Ca^{2+}-ATPase activity is inhibited by phenylhydrazine. Since monovalent ions participate in colloid-osmotic lysis and since denatured and/or oxidized hemoglobin binds to band 3, we have tested the hypothesis that oxidants affect anion translocation. The inhibition of anion translocation described below may contribute to the oxidative disruption of erythrocyte function and cellular integrity.

Materials and Methods

Materials

NBD-taurine (*N*-(2-aminoethylsulfonate)-7-nitrobenz-2-oxa-3-diazole) was purchased from Molecular

Correspondence: H.R. Petty, Department of Biological Sciences, Wayne State University, Detroit, MI 48202, U.S.A.

Probes Inc. (Eugene, OR). The physical properties of this reagent have been previously described [18]. DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid) was obtained from Molecular Probes and Sigma Chemical Co. (St. Louis, MO).

Erythrocytes

Fresh human erythrocytes were obtained from adult volunteers by a finger prick. Cells were suspended in PBS then washed twice with buffer.

Continuous monitoring of transport by fluorescence (CMTF)

Anion transport was measured by the CMTF method, as previously described [18–21]. Erythrocytes were equilibrated with the fluorescent anion NBD-taurine (5 mM) for 2 to 3 h at 37°C or for 16 h at 4°C followed by 2–3 h at 37°C. Cells were placed on ice then rapidly washed three times with ice-cold buffer using a Beckman microfuge. Due to quenching by hemoglobin, NBD-taurine-loaded erythrocytes have little or no fluorescence. As time passes, the efflux of NBD-taurine leads to a time-dependent increase in fluorescence intensity. After washing at 4°C, 20 μl of cells ($2.25 \cdot 10^9$/ml) were added to transport buffer (250 mM sucrose, 15 mM KCl, 25 mM K_2PO_4 (pH 7.2)) to a total volume of 3.5 ml held at 37°C in thermostated sample holder. Upon mixing the time-dependent changes in fluorescence intensity were recorded with a Perkin-Elmer (Norwalk, CT) MPF-66 fluorometer interfaced to a Perkin-Elmer 7300 computer. The excitation and emission monochrometers were set at 468 nm and 545 nm with slit widths of 4 and 10 nm, respectively. The initial fluorescence intensity is F_0 and the final (or infinite time) value of the fluorescence intensity is F_∞. Intermediate values of the fluorescence intensities at times t are given as $F(t)$. The internal concentration of NBD-taurine was determined by fluorometry. Briefly, NBD-taurine labeled cells were washed at 4°C then lysed with distilled H_2O. Trichloroacetic acid (Sigma) was added at a final concentration of 4% by volume to precipitate protein. The fluoresence intensity of the supernate was compared to an NBD-taurine calibration curve to determine the NBD-taurine concentration. The DIDS control experiments were performed after equilibration with NBD-taurine. Cells, in the presence of NBD-taurine and transport buffer, were incubated with a final concentration of 50 μM DIDS for 30 min at room temperature. The cells were washed in the cold then suspended in transport buffer.

Data analysis

The data were quantitatively analyzed to yield the kinetic rate constant, k, of efflux. During the experiments described below the initial internal concentration of NBD-taurine was much less than its apparent Michaelis constant. The data were therefore analyzed in the trace mode, which is particularly well-suited for ion translocation inhibition studies [19,21]. The rate of efflux is

$$k(t) = \frac{\mathrm{d}[F_\infty - F(t)]}{\mathrm{d}t} \tag{1}$$

where $k(t)$ is the instantaneous rate parameter [19]. The rate constant can be extracted by several means [21].

The value of k was obtained using three approaches. By measuring the initial slope of the efflux curve $(\Delta F/\Delta t)_i$, the rate constant was calculated from

$$k = \frac{(\Delta F/\Delta t)_i}{A} \tag{2}$$

where

$$A = F_\infty - F_0. \tag{3}$$

as described by Eidelman and Cabantchik [19]. The kinetic rate constant of NBD-taurine efflux was also calculated by an iterative nonlinear analysis of data. The equation

$$F(t) = F_\infty - A\,\mathrm{e}^{-kt} \tag{4}$$

was fit. Approximately 30 data points of each data were included in each iterative analysis. In the third method of analysis the $\ln[(F_\infty - F_i)/A]$ was plotted against t. Time points beyond 20 min were not employed due to their increased relative error. The value of k can be determined by a least-squares analysis of the slope [21]. Since this line is known to pass through the origin and any errors are in the y coordinates, the sum of the squared residuals of the equation $y = mx$, where m is the slope, were minimized according to:

$$m = \frac{\sum_{i=1}^{n} x_i y_i}{\sum_{i=1}^{n} x_i^2} \tag{5}$$

where n = the number of points [22]. Comparable results were obtained by these three methods.

Statistical treatment of data

The results are expressed as means ± S.E. Data were analyzed using an Apple IIe computer and stats plus software (Human Systems Dynamics, Northridge, CA).

Optical microscopy

Optical micrographs were recorded using an Axiovert microscope with a 100 × oil immersion objective (Carl Zeiss, New York, NY). The image was collected on a

CCD camera. The video signal was displayed on a 19 inch monitor. Heinz bodies were stained with 2% crystal violet in PBS for 10–15 min. [23]. Micrographs were recorded using a Polaroid video printer.

Scanning electron microscopy

Scanning electron microscopy was performed as we have previously described [24]. Briefly, cell populations were fixed in suspension with 2% glutaraldehyde in PBS for 1 h at room temperature. The samples were thoroughly washed then post-fixed for 1 h in 1% OsO_4 in PBS or transport buffer at room temperature. The cells were dehydrated using a graded series of ethanol washes followed by three washes in absolute ethanol. Specimens were placed in baskets constructed from BEEM capsules using solvent-resistant Nucleopore filters (Pleasanton, CA) to prevent cell loss. Cells were then critical point dried in an Omar spc-1500 critical point dryer (Bomar Co., Tacoma, WA) using freon 113 as the transition fluid. Samples were observed and photographed using a Philips 505 scanning electron microscope operated at 30 kV.

Results

Cell morphology

Fig. 1 shows the effect of phenylhydrazine on erythrocyte morphology. Erythrocytes ($6 \cdot 10^7$/ml) in PBS or transport buffer were incubated in the absence (Fig. 1A) or presence (Fig. 1B) of 5 mM phenylhydrazine for 5 min at room temperature. The samples were then washed with buffer and fixed. Indistinguishable results were obtained when PBS was replaced with transport buffer. Normal erythrocytes displayed a typical biconcave shape. However, in the presence of phenylhydrazine many pseudopod-like extensions of the plasma membrane were formed (Fig. 1B). These extensions may be due to weakened membrane–cytoskeleton interactions.

Heinz body formation is a common feature of many hemoglobin disorders and oxidant or drug treatment [25]. Fig. 2 confirms the formation of phenylhydrazine-induced Heinz bodies. Erythrocytes were incubated with 5 mM phenylhydrazine for 5 min at room temperature. The cells were then stained with crystal violet. Numerous Heinz bodies can be observed within the erythrocytes. The formation of membrane protuberances (Fig. 1B) and Heinz bodies (Fig. 2B) clearly precede cytolytic rupture of erythrocytes.

Effects of phenylhydrazine on fluorescence emission of NBD-taurine and erythrocytes

The effects of phenylhydrazine on the fluorescence properties of NBD-taurine and erythrocytes were measured. This controls for possible changes in the total fluorescence intensity due to the phenylhydrazine. Table I lists the fluorescence intensity ± standard deviation for four combinations of buffer, NBD-taurine, erythrocytes, and 0 to 10 mM phenylhydrazine. These experiments were performed under conditions that were identical to those of the transport assays described below. As can be seen in row A, the addition of phenylhydrazine to transport buffer had no effect on the buffer's minimal level of fluorescence emission. Phenylhydrazine was added to 15 µl of intact unlabeled erythrocytes ($6 \cdot 10^7$/ml) in suspension (Table I, row B). A small but significant dose-dependent increase in the fluorescence intensity is observed. Phenylhydrazine has been previously reported to induce the formation of fluorescent Heinz bodies in red cells [26]. The small increase in fluorescence observed here likely arises from this source. Since phenylhydrazine is an oxidant, we have examined its ability to influence NBD-taurine fluorescence. Table I, row C shows the effect of phenylhydrazine treatment on the emission intensity of 0.8 µM NBD-taurine. At 10 mM phenylhydrazine, the emission of NBD-taurine is decreased by 13% under these conditions. To further probe the effect of phenylhydrazine on

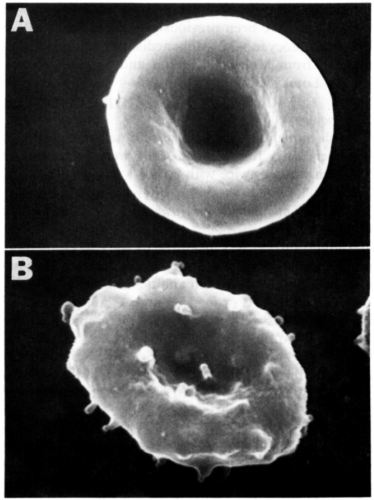

Fig. 1. Scanning electron micrographs of erythrocytes are shown. Cells were prepared for electron microscopy as described in 'Materials and Methods'. Representative micrographs of erythrocytes treated for 5 min with: (A) buffer alone (×8840) and (B) 5 mM phenylhydrazine (×9265) are shown.

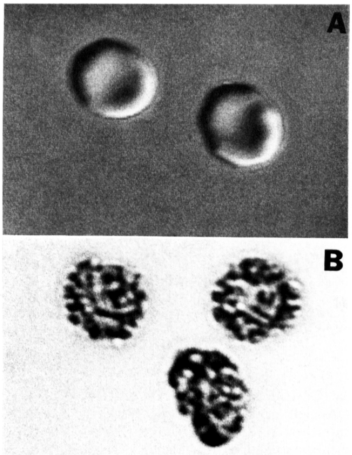

Fig. 2. Optical micrographs of human erythrocytes in the absence and presence of Heinz bodies. Panel A shows a differential interference contrast micrograph of human erythrocytes incubated PBS for 5 min (×2640). Panel B shows cells incubated for 5 min with 5 mM phenylhydrazine, stained with crystal violet, then examined by bright-field microscopy. Heinz bodies are observed in the phenylhydrazine-treated sample (×2640).

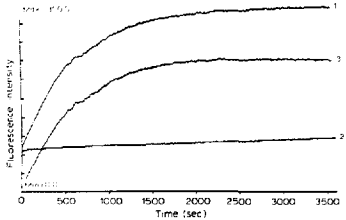

Fig. 3. The kinetics of NBD-taurine efflux from control cells is shown. The ordinate gives the fluorescence intensity (arbitrary units) and the abscissa lists the time (s). Trace 1 shows a representative efflux curve. Trace 2 shows a matched sample with DIDS inhibition of anion translocation. Trace 3 shows the corrected subtraction curve (trace 1 − trace 2).

fluorescence emission, combined experiments utilizing NBD-taurine, erythrocytes, and phenylhydrazine were performed. Erythrocytes were first loaded with NBD-taurine, as described above. The cells were washed then treated with various concentrations of phenylhydrazine at 4°C. The cells were then lysed using distilled H_2O. Proteinaceous material was precipitated using trichloroacetic acid (final concn. 4% vol./vol.). The fluorescence intensities of the supernates were then measured. Table I, row D shows the dose-dependent decrease in fluorescence intensity of this sample (means ± S.D.). A maximal decrease of 26% is observed. The greater decrease in the presence of erythrocytes is likely due to the ability of phenylhydrazine to interact with hemoglobin to form superoxide anions [6]. These radicals, in turn, can oxidize some fluorochromes.

Efflux of NBD-taurine

The efflux of NBD-taurine was followed by the CMTF technique [18]. Fig. 3 shows representative tracings of NBD-taurine efflux from erythrocytes in transport buffer. Trace 1 gives the time-dependent increase in fluorescence due to probe efflux from cells at 37°C. The appearance of these data is in agreement with that of previous investigators [18,19]. Trace 2 shows a matched experiment utilizing DIDS treatment. NBD-taurine-equilibrated cells were mixed with 50 μM DIDS for 30 min then washed. During these conditions NBD-taurine leakage was not significant. No significant change was observed during the time course of this experiment. This confirms that NBD-taurine efflux is mediated by band 3. The apparent fluorescence intensity of the DIDS-inhibited erythrocytes is above zero, as reported by previous investigators [18]. This is due to several factors such as light scattering by the erythrocytes and trace quantities of NBD-taurine absorbed to

TABLE I

Effect of phenylhydrazine on the emission of NBD-taurine

$n = 4$. $\lambda_{em} = 545$ nm. $\lambda_{ex} = 468$ nm; emission and excitation slit widths were 4 nm and 10 nm, respectively.

Condition	Fluorescence intensities with phenylhydrazine concentrations (mM)				
	buffer only	0.5	1	5	10
A. phenylhydrazine only	4.65 ± 0.27	4.10 ± 0.37	4.32 ± 0.44	4.20 ± 0.48	4.14 ± 0.58
B. phenylhydrazine + RBCs	4.04 ± 1.87	7.15 ± 0.38	8.82 ± 1.88	11.1 ± 4.8	18.6 ± 11.2
C. 0.8 μM NBD-taurine + phenylhydrazine	332 ± 7	319 ± 2	313 ± 6	301 ± 8	290 ± 7
D. NBD-taurine-labeled RBCs + phenylhydrazine	381 ± 30	333 ± 16	303 ± 22	272 ± 13	262 ± 16

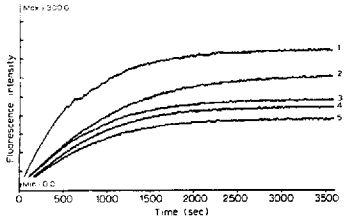

Fig. 4. The time-dependence of NBD-taurine efflux from control and phenylhydrazine-treated erythrocytes is shown. The fluorescence intensity and time are given at the ordinate and abscissa, respectively. Traces 1–5 show samples treated with 0, 1, 2.5, 5, and 10 mM phenylhydrazine, respectively.

the erythrocyte surface or remaining after washing. Although this spurious but constant contribution has no effect upon the kinetic analysis, we can eliminate this factor by subtracting Trace 2 from Trace 1. When this is performed, Trace 3 is obtained. These data, which are specific for band 3, were then analyzed as described in Materials and Methods. All experiments utilized DIDS controls in this fashion. When a number of independent measurements were analyzed ($n = 9$), the mean value of k was found to be -0.090 ± 0.017 min^{-1} with a range of -0.065 to -0.115.

Effect of phenylhydrazine on NBD-taurine efflux

Fig. 4 shows representative CMTF tracings of NBD-taurine efflux from erythrocytes treated with 0, 1, 2.5, 5, and 10 mM phenylhydrazine (traces 1 through 5, respectively). Both the initial slopes and time-dependent intensities of NBD-taurine efflux are changed. Although the phenylhydrazine-treated samples are roughly similar, the 10 mM treatment has the greatest effect. The values of F_∞ were determined after cell lysis and TCA precipitation, as described above. To extract k from these data, they were analyzed as described in the Materials and Methods. Fig. 5 shows representative plots of $-\ln[(F_\infty - F_t)/A]$ versus time which illustrate the effect of phenylhydrazine on anion transport. The straight lines of Fig. 5 indicate that the value of the rate constant during control or phenylhydrazine treatment experiments is a time-independent quantity. The control and 10 mM phenylhydrazine experiment were chosen to illustrate this effect. The value of k is also determined from the slope of these lines. In these two particular cases the values of k are -0.104 min^{-1} and -0.0625 min^{-1} for control and phenylhydrazine-treated samples, respectively.

Fig. 6 shows the quantitative dose-dependent effects of phenylhydrazine on NBD-taurine efflux for all experiments. Phenylhydrazine experiments were accompanied by matched controls. The effects of phenylhydrazine on k are shown as a % of the value of matched control experiments. This corrects for a small but significant variability in k among individuals. The difference in k between control and phenylhydrazine treatments of ≥ 1 mM were statistically significant. Concentrations < 1 mM gave a statistically insignificant level of inhibition. At 10 mM phenylhydrazine the value of k is decreased to just under one-half of its control value. The reduction in k is maximal by 2.5 mM phenylhydrazine since this point is not significantly different from 10 mM phenylhydrazine. This dose-dependence correlates with the ability of phenylhydrazine to induce Heinz bodies (Fig. 2B).

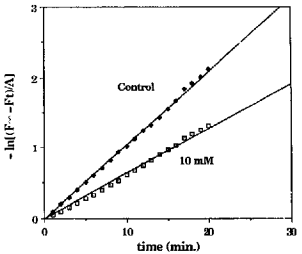

Fig. 5. Kinetic analyses of NBD-taurine are shown. Control (■) samples and erythrocytes treated with 10 mM phenylhydrazine (□) are shown. The value of $-\ln[(F_\infty - F_t)/A]$ is plotted against time (min). The solid lines represent the least-squares analysis of each data set. The decrease in slope of the treated sample shows that k is diminished.

Although the samples and controls were treated in an identical fashion, it is possible that the results were affected by the transport buffer's composition. To con-

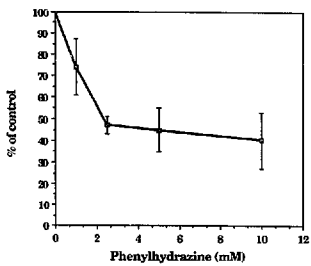

Fig. 6. The dose-dependent effects of phenylhydrazine on anion transport are shown. The value of k expressed as a % of control is plotted against phenylhydrazine concentration.

trol for its low Cl^- composition, experiments were conducted in Tris-buffered saline (TBS; 20 mM Tris, 147 mM NaCl (pH 7.4)). The magnitude of k in TBS was significantly reduced to 0.0285 min^{-1} ($n = 3$). This effect of Cl^- on NBD-taurine transport has been previously described [18]. Treatment with 10 mM phenylhydrazine as described above resulted in a $46 \pm 13\%$ inhibition of k in TBS. This degree of anion transport inhibition in indistinguishable from that shown in transport buffer (Fig. 6). Although the buffer content affects the magnitude of k, it does not influence the relative effects of phenylhydrazine.

Discussion

Phenylhydrazine directly interacts with hemoglobin leading to the formation of superoxide anions, hydrogen peroxide and Heinz bodies [6]. Phenylhydrazine and compounds derived from its action may influence membrane function. Previous studies have focused attention on the ability of phenylhydrazine to disturb transmembrane cation distributions [16,17]. Although anion translocation is required for erythrocyte function, the possible relationship between anion translocation and oxidant damage or Heinz body formation have not been previously explored. In the present study we have shown that the oxidative drug phenylhydrazine inhibits anion translocation of human erythrocytes.

Our results indicate that the CMTF technique [18–21] can be used in diverse studies of anion transport. By carefully controlling for the effects of drug addition, the fluorescence signal can be quantitatively understood. In addition, DIDS was found to completely block NBD-taurine efflux thus confirming that it escapes via band 3. In parallel experiments with and without DIDS, the effects of light scattering, non-specific absorption, and non-specific loss of NBD-taurine could be easily controlled for using spectral subtraction. The DIDS-treated sample was subtracted from the untreated sample yielding the anion translocation curve. Our results with the CMTF technique during control conditions are in good agreement with previous studies of NBD-taurine during similar conditions [18–21]. The values of k reported for NBD-taurine (see, for example, Ref. 18) is within the range we report.

Our studies have shown that the oxidative drug phenylhydrazine diminishes the rate constant for NBD-taurine efflux. At phenylhydrazine concentrations of ≥ 2.5 mM, the value of k is diminished to less than one-half of its control value. The straight lines of Fig. 5 show that k is a time-independent quantity. In this case the different slopes of these lines show that k has decreased in absolute value from -0.104 min^{-1} to -0.0625 min^{-1}. The dose-dependent effects of many studies are summarized in Fig. 6. Similar results were achieved by the three methods of trace analysis given above. Furthermore, phenylhydrazine's relative level of inhibition was indistinguishable in both low- and high-Cl^- buffers. This is important since the Cl^- gradient during efflux experiments could potentially affect a cell's response to phenylhydrazine. Our results suggest that phenylhydrazine's inhibition of anion transport is independent of buffer conditions. The inhibition of anion transport is likely due to an alteration in the function of the erythrocyte membrane protein band 3. This seems fairly certain since: band 3 is principally responsible for anion transport and NBD-taurine efflux is specific for band 3 as judged by DIDS treatment.

A variety of factors may participate in the phenylhydrazine-mediated inhibition of transport. These may include both direct and indirect mechanisms. For example, the direct oxidation of band 3 could affect anion transport. Another possibility is that Heinz bodies directly inhibit anion translocation by binding to band 3. Heinz bodies are known to bind to band 3 in human erythrocytes [10,27]. When band 3's N-terminal is disturbed by Heinz bodies, a conformational change may be induced in its large transmembrane domain resulting in diminished anion translocation. Moreover, such a conformational change could also be induced by the ability of Heinz bodies to cluster band 3 in membranes [9,11]. In addition to direct effects on band 3's structure, phenylhydrazine could also affect anion translocation via indirect routes. In particular, phenylhydrazine has been reported to alter lipid packing and decrease membrane fluidity [13,14]. Since membrane transport proteins are sensitive to membrane fluidity (see, for example, Ref. 28), the phenylhydrazine-mediated reduction in fluidity may also diminsh anion translocation.

The inhibition of anion translocation observed in the present study may participate in several physiological processes. For example, reduced anion transport may contribute to the pathology of genetic or acquired erythrocyte deficiencies. Previous studies [29] have indicated that superoxide anions can cross erythrocyte membranes via the DIDS-sensitive anion channel. Unpublished studies from this laboratory indicate that DIDS decreases superoxide-mediated specific cytolysis by 50% (Zhou, M. and Petty, H.R., unpublished data). Therefore, phenylhydrazine may be slowly hemolytic because its early oxidative effects diminish the rate of anion translocation. The diminished anion translocation may adversely affect oxygen transport and survival of erythrocytes.

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