

# Decreased Survival In Vivo of Diamide-incubated Dog Erythrocytes

## A MODEL OF OXIDANT-INDUCED HEMOLYSIS

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**ABSTRACT** Erythrocytes from patients with chronic hemolytic variants of glucose-6-phosphate dehydrogenase (G-6-PD) deficiency have structural membrane protein abnormalities accompanied by decreased cell membrane deformability which we postulate represent the consequences of oxidant-induced membrane injury. To evaluate the pathophysiologic significance of oxidant-induced membrane injury, we studied the in vitro and in vivo effects of the thiol-oxidizing agent, diamide, on dog erythrocytes. In vitro incubation of dog erythrocytes with 0.4 mM diamide in Tris-buffered saline for 90 min at 37°C resulted in depletion of GSH, formation of membrane polypeptide aggregates (440,000 and >50,000,000 daltons) and decreased cell micropipette deformability, abnormalities similar to those observed in the erythrocytes of patients with chronic hemolytic variants of G-6-PD deficiency. In addition, diamide-incubated cells had increased viscosity and increased membrane specific gravity, but no change in ATP. Reinjection of <sup>51</sup>Cr-labeled, diamide-incubated cells was followed by markedly shortened in vivo survival and splenic sequestration. Further incubation of diamide-incubated cells in 4 mM dithiothreitol reversed the membrane polypeptide aggregates, normalized micropipette deformability, decreased cell viscosity, prolonged in vivo survival, and decreased splenic sequestration.

These studies demonstrate that diamide induces a partially reversible erythrocyte lesion which is a useful model of oxidant-induced membrane injury. They sug-

gest that oxidant-induced erythrocyte membrane injury plays an important role in the pathophysiology of chronic hemolysis which accompanies some G-6-PD variants.

## INTRODUCTION

Previous studies in our laboratory demonstrated that erythrocytes from patients with chronic hemolytic variants of glucose-6-phosphate dehydrogenase (G-6-PD)<sup>1</sup> deficiency had structural membrane protein abnormalities that were accompanied by decreased cell deformability (1, 2). Erythrocyte membranes from patients with chronic hemolytic variants of G-6-PD deficiency contained polypeptide aggregates composed of disulfide-linked spectrin dimers and heteropolymers (1, 2). Similar aggregates were not observed in erythrocytes from patients with G-6-PD deficiency not accompanied by chronic hemolysis (2). GSH concentrations were reduced, but ATP was normal; therefore, we postulated that the membrane aggregates were the consequence of oxidant-induced formation of disulfide bonds (2). Since micropipette deformability was also decreased in the erythrocytes of these patients, chronic hemolysis was attributed to the oxidant-induced membrane abnormality (2). Subsequent studies demonstrated increased density of a subpopulation of erythrocyte membranes obtained from patients with chronic hemolytic G-6-PD variants.<sup>2</sup> To confirm our postulates regarding the pathophysiologic significance of the demonstrated erythrocyte membrane abnormality, we

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<sup>1</sup>Abbreviation used in this paper: G-6-PD, glucose-6-phosphate dehydrogenase.

<sup>2</sup>Flynn, T. P., G. J. Johnson, and D. W. Allen. Sucrose density gradient analysis of erythrocyte membranes in hemolytic anemias. In preparation.

sought an *in vivo* model of oxidant-induced erythrocyte membrane injury.

Diamide (diazenedicarboxylic acid bis [*N,N*-dimethylamide], II), a thiol-oxidizing diazene derivative, depletes erythrocyte GSH without forming Heinz bodies (3), thus simulating the erythrocytes of unsplenectomized patients with chronic hemolytic G-6-PD variants. In addition, diamide treatment of human erythrocytes results in decreased shear elasticity (4). Therefore, we studied the effects of diamide on dog erythrocytes *in vitro*, and observed the effect of diamide incubation on dog erythrocyte survival *in vivo*.

## METHODS

Healthy mongrel dogs with normal hematocrits were studied. Normal erythrocyte survival was documented (method described below) in each animal before study of diamide-incubated erythrocyte survival.

***In vitro studies.*** Blood was obtained by jugular venipuncture and immediately added to sodium heparin in sterile glass tubes. 30 ml of heparinized whole blood was incubated with 50  $\mu$ Ci (or 75  $\mu$ Ci if the dog had previously received  $^{51}\text{Cr}$ )  $\text{Na}_2\text{[}^{51}\text{Cr]O}_4$  (Chromitope, E. R. Squibb & Sons, Princeton, N. J.) for 30 min. Following centrifugation, the labeled erythrocytes were washed twice in sterile normal saline and incubated with either 0.2 or 0.4 mM diamide (Sigma Chemical Co., St. Louis, Mo.) in 9 vol of Tris-buffered saline or with Tris-buffered saline alone for 90 min at 37°C. In certain experiments  $^{51}\text{Cr}$ -labeled erythrocytes incubated with 0.4 mM diamide were centrifuged and washed once in phosphate-buffered saline, pH 7.4. The erythrocytes were then suspended in an equal volume of the animal's own heparinized plasma plus 7 vol of phosphate-buffered saline to which 1 vol of 40 mM dithiothreitol (final concentration 4 mM) was added. The cells were incubated in 4 mM dithiothreitol for 30 min at 37°C. Erythrocyte membranes were prepared in cold room at 0°–4°C with methods described by Dodge et al. (5), and by Fairbanks et al. (6). Polyacrylamide-gel electrophoresis in sodium dodecyl sulfate was performed by a modification (7) of the method of Fairbanks et al. (6). Before electrophoresis, membrane samples were heated in 1% sodium dodecyl sulfate at 100°C for 2 min. Membranes were applied to gels containing 4% acrylamide and run for 5 h. The gels were stained with Coomassie Blue and scanned. Membrane polypeptide aggregates at the origin of the gel (>50,000,000 daltons) and those of 440,000 daltons were estimated by planimetry in terms of the amount of other protein present on the gels (bands 1, 2, 2.1, 2.2, and 2.3). A correction for the meniscus of gels without aggregates at the origin was used for the planimetry results from scans of the high molecular weight aggregates at the origin. Erythrocyte G-6-PD (World Health Organization method), GSH, and ATP were determined by the methods of Beutler (8). Membrane deformability was measured by the micropipette aspiration technique of Dreher et al. (9). Membrane density was evaluated by measuring the specific gravity of once-washed membranes placed on 32 ml 15–60% linear sucrose gradients in 10 mM Tris and 1 mM EDTA at pH 7.4 and centrifuged 75,000 *g* for 16 h. 1-ml fractions were collected; absorbancy was measured at 280 nm, and the specific gravity was determined by refractometry. Erythrocyte viscosity was measured in a Brookfield LVT cone plate microviscometer (Brookfield Engineering Laboratories, Inc., Stoughton, Mass.). Erythrocytes were washed three times in phosphate-buffered saline and suspended in phosphate-buffered saline with 0.5% bovine serum albumin. Following

sedimentation to 80% packed volume, erythrocyte viscosity was determined at 37°C at multiple shear rates using the CP 7 and CP 7A spindles.

***In vivo studies.*** Erythrocyte survival *in vivo* was determined by a modification of the  $^{51}\text{Cr}$  labeling technique of Mollison and Veall (10). The  $t_{1/2}$  of labeled, reinjected erythrocytes was determined from a log/linear plot of whole blood radioactivity.

Following injection of  $^{51}\text{Cr}$ -labeled cells, blood samples were drawn at 5, 10, 20, 30, 60, 120, and 180 min and, thereafter, at 24-h intervals. The blood was added to dipotassium EDTA, and whole blood radioactivity was determined in a liquid scintillation counter after freeze-thaw induced hemolysis. Background radioactivity of a blood sample, obtained before  $^{51}\text{Cr}$  labeling and corrected for isotopic decay, was subtracted from each postinjection sample. Elution of  $^{51}\text{Cr}$  from labeled erythrocytes was evaluated by incubating these cells in autologous plasma at 37°C and sequentially determining plasma radioactivity. To evaluate the contribution of intravascular hemolysis, the postinjection blood samples were immediately centrifuged, with the plasma and red blood cells counted separately in five studies, and plasma hemoglobin determined spectrophotometrically in three studies. Splenic sequestration was evaluated by external probe counting, using a spectrometer with a medium energy sodium iodide crystal (model 2200, Ludlum Measurements, Inc., Sweetwater, Texas), over the spleen, liver, and heart just before each post-injection venipuncture.

***Statistical methods.*** Standard statistical methods were used to calculate standard deviations and *t* tests of significance (11). The results are expressed as the mean  $\pm$  1 SD.

## RESULTS

***In vitro studies.*** Erythrocyte GSH was depleted by *in vitro* incubation with 0.4 mM diamide but not 0.2 mM diamide (Table I). Erythrocyte ATP was unchanged from buffer control values following incubation with either concentration of diamide (Table I). Membrane polypeptide aggregates of 440,000 and >50,000,000 daltons were observed in cells incubated with 0.4 mM diamide, but not in cells incubated with 0.2 mM diamide (Fig. 1). Quantitative evaluation of these membranes by planimetry revealed ~5–10% of the total membrane protein in each of the two molecular

TABLE I  
Dog Erythrocyte GSH and ATP Following *In Vitro*  
Incubation with Diamide or Buffer

Dog	Diamide	GSH	ATP
	mM	$\mu\text{mol/g Hb}^*$	$\mu\text{mol/g Hb}$
1	0	7.37	1.27
2	0	7.10	1.29
2	0.2	7.01	1.17
3	0.2	7.11	1.64
1	0.4	0.47	1.30
2	0.4	0.22	1.51
4	0.4	0.20	1.43
5	0.4	0.00	1.29

\* Hemoglobin.

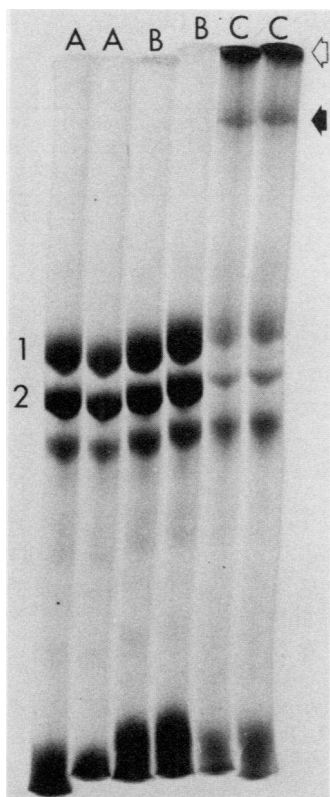


FIGURE 1 Polyacrylamide gel (4%) electrophoresis in sodium dodecyl sulfate of dog erythrocyte membranes from buffer-incubated cells (A), 0.2 mM diamide-incubated cells (B), and 0.4 mM diamide-incubated cells (C). Bands 1 and 2 and polypeptide aggregates of >50,000,000 daltons ( $\Rightarrow$ ) and 440,000 daltons ( $\blacktriangleright$ ) are labeled. The photograph accentuates the aggregates at the origin, but densitometric scans indicate that the aggregates are approximately equal in quantity (9.9 vs. 8.7% of total membrane protein, respectively).

weight aggregates (Table II). Heinz bodies were not present after incubation with either concentration of diamide. Despite the absence of aggregates in membranes incubated with 0.2 mM diamide erythrocyte micropipette deformability was decreased as it was in cells incubated with 0.4 mM diamide (Table II). Erythrocyte viscosity measured at multiple shear rates was not changed by incubation with 0.2 mM diamide. However, cells incubated with 0.4 mM diamide demonstrated significantly increased viscosity at all shear rates measured, as well as increased membrane density (Table III). Loss of  $^{51}\text{Cr}$  from labeled cells incubated in autologous plasma was slightly greater in diamide-incubated than in buffer-incubated erythrocytes. In the plasma of diamide-incubated cells after 2 h incubation at 37°C, only 8% of initial erythrocyte radioactivity was found, compared with 3% for buffer-incubated cells. The increase in plasma radioactivity observed during in vitro incubation was primarily attributable to hemolysis, since the plasma hemoglobin of diamide-incubated cells rose from 625 mg/dl initially to 1,375 mg/dl after 120 min incubation at 37°C in autologous plasma. Cells incubated in buffer under the same conditions had a rise of plasma hemoglobin from 335 to 770 mg/dl.

All in vitro erythrocyte abnormalities acquired by incubation of erythrocytes in 0.4 mM diamide were fully or partially reversed by further incubation in dithiothreitol (Table IV). 2% of initial erythrocyte radioactivity was recovered from the plasma of cells incubated with diamide followed by dithiothreitol after 2 h of in vitro incubation at 37°C. Plasma hemoglobin rose from 49 to 325 mg/dl during this study.

*In vivo studies.* Normal  $^{51}\text{Cr}$ -labeled erythrocyte survival ( $t_{1/2}$  23–27 d) was documented in each animal before study of diamide-incubated cell survival. A

TABLE II  
Membrane Polypeptide Aggregates and Cell Deformability Following Incubation of Dog Erythrocytes with Diamide or Buffer

Dog	Diamide	Membrane polypeptide aggregates		Erythrocyte deformability		Pipette size	Pressure
		>50,000,000 daltons	440,000 daltons	Pre-incubation	Post-incubation		
	mM	% membrane protein		$\mu\text{m}$ tongue extension		$\mu\text{m}$	cm water
1	0	0	0	11.8 $\pm$ 0.3	12.1 $\pm$ 0.3	1.8	-25
2	0	0	0	10.2 $\pm$ 0.4	10.5 $\pm$ 0.6	1.8	-25
1	0.2	0	0	13.2 $\pm$ 0.3	10.0 $\pm$ 0.6*	2.0	-25
3	0.2	0	0	11.6 $\pm$ 0.4	7.2 $\pm$ 0.3*	1.8	-25
1	0.4	5.0	5.4	13.2 $\pm$ 0.3	9.6 $\pm$ 0.5*	2.0	-25
2	0.4	7.2	5.1	10.5 $\pm$ 0.6	5.8 $\pm$ 0.4*	2.0	-25
4	0.4	9.9	8.7	3.1 $\pm$ 0.3	2.3 $\pm$ 0.4*	1.0	-50
5	0.4	5.3	7.9	3.2 $\pm$ 0.4	2.1 $\pm$ 0.4*	1.0	-50

\* Significantly different from preincubation values ( $P < 0.001$ ).

**TABLE III**  
*Viscosity and Membrane Specific Gravity of Dog Erythrocytes Following Incubation with Diamide or Buffer*

		Diamide		
		0	0.2	0.4
		<i>mM</i>		
Viscosity shear rate, $s^{-1}$	90	9.29±0.20*	7.57±0.01	17.56±0.12‡
	45	12.34±1.62	9.85±0.39	22.62±0.36‡
	22.5	14.20±0.51	13.13±1.18	29.63±1.31‡
	11.25	21.39±0.43	20.46±1.30	44.16±5.52‡
Membrane specific gravity		1.1439±0.0008*	1.1416±0.0006	1.1538±0.0002‡

\* Four determinations performed on one sample.

‡ Significantly greater than buffer-incubated controls ( $P < 0.005$ ).

minimum of 30 d elapsed between the  $t_{1/2}$  of the original control survival study and the subsequent diamide-incubated survival. Following the injection of washed cells incubated in Tris-buffered saline for 90 min, blood

radioactivity decreased  $21 \pm 3.0\%$  (three studies) during the first 60 min. This rapid initial decrease in radioactivity was apparently due to hemolysis of cells injured during incubation. Inasmuch as whole blood and

**TABLE IV**  
*In Vitro and In Vivo Effects of Incubation of Dog Erythrocytes with 0.4 mM Diamide or 0.4 mM Diamide Followed by 4 mM Dithiothreitol*

		Results	
		Diamide-incubated	Diamide-incubated followed by dithiothreitol-incubated
Membrane aggregates, %			
<i>membrane protein</i>			
>50,000,000 daltons		5.2	0.0
440,000 daltons		4.0	0.4
Viscosity shear rate, $s^{-1}$	90	17.85±0.06	12.87±0.02
	45	23.41±0.18	15.76±0.04
	22.5	37.52±2.04	19.95±0.29
	11.25	64.56±0.29	28.28±0.43
RBC* deformability, $\mu M$ tongue extension‡		7.9±1.9	11.2±0.9
Membrane specific gravity		1.1490±0.0011	1.1460±0.0010
RBC radioactivity following reinjection, % of initial count			
60 min		11	62
180 min		2	46
Splenic sequestration, ratio-spleen:heart			
180 min following reinjection		9.5	1.8
Plasma hemoglobin, mg/dl			
10 min following reinjection		28	13
120 min following reinjection		14	16
Urinary $^{51}Cr$ excretion, % in 24 h following reinjection		2.0	1.9

\* Erythrocytes

‡ 1.8  $\mu m$  pipette; -25 cm  $H_2O$  pressure.

erythrocyte radioactivity were approximately equal, it is likely that the greater part of this early hemolysis occurred in extravascular sites, although no significant splenic accumulation of radioactivity was detected. After 24 h the survival curve paralleled that of the normal survival curve obtained with unincubated cells. Incubation with 0.4 mM diamide resulted in a marked immediate fall in blood radioactivity followed by a second component of the survival curve with a lower slope. A representative study is illustrated in Fig. 2. 60 min after the injection of 0.4 mM diamide-incubated cells,  $88.8 \pm 6.7\%$  (six studies) of the isotope was cleared from the blood. Simultaneous external counting revealed marked splenic sequestration of  $^{51}\text{Cr}$ -labeled cells (Fig. 2). The mean ratio between the spleen and heart was  $5.2 \pm 2.7$  in five studies. Erythrocytes incubated with 0.2 mM diamide demonstrated a slight initial decrease in radioactivity similar to that observed in the controls, and no significant splenic sequestration was observed (Fig. 2). The  $t_{1/2}$  of the second component of the curve was 19.6 and 22 d for two studies of 0.2 mM diamide-incubated cells. Separate counting of erythrocytes and plasma, centrifuged immediately after venipuncture, revealed a higher proportion of radioactivity in the plasma following incubation with 0.4 mM diamide than following incubation in buffer, but the clearance of plasma radioactivity after reinjection of diamide-incubated cells did not differ from that of the saline-incubated control. After reinjection of 0.4 mM diamide-incubated cells, 17% of the total reinjected radioactivity was present in plasma at 60 min and 8% after 180 min. Plasma hemoglobin rose to a maximum of 28 mg/dl, 10 min after reinjection of 0.4 mM diamide-incubated erythrocytes. 2–5% of the injected radioactivity was recovered during the first 24 h from the urine of dogs injected with cells incubated with 0.4 mM diamide (three studies), whereas 0–0.6% was recovered from the urine of dogs injected with cells incubated with 0.2 mM diamide (two studies) or saline (one study).

Reinjection of dog erythrocytes incubated with 4 mM dithiothreitol alone resulted in a  $t_{1/2}$  of 15 d. Incubation of 0.4 mM diamide-incubated cells with dithiothreitol resulted in significantly less hemolysis after reinjection than that observed after incubation with diamide alone (Table IV). The initial phase of the two-component survival curve had a  $t_{1/2}$  of 120 min, whereas the second phase had a  $t_{1/2}$  of 44 h.

## DISCUSSION

In vitro incubation of human erythrocytes with diamide induces abnormalities similar to those we have observed in patients with G-6-PD variants accompanied by chronic hemolysis (1, 2). Previous studies demonstrated that diamide lowered intracellular GSH, with-

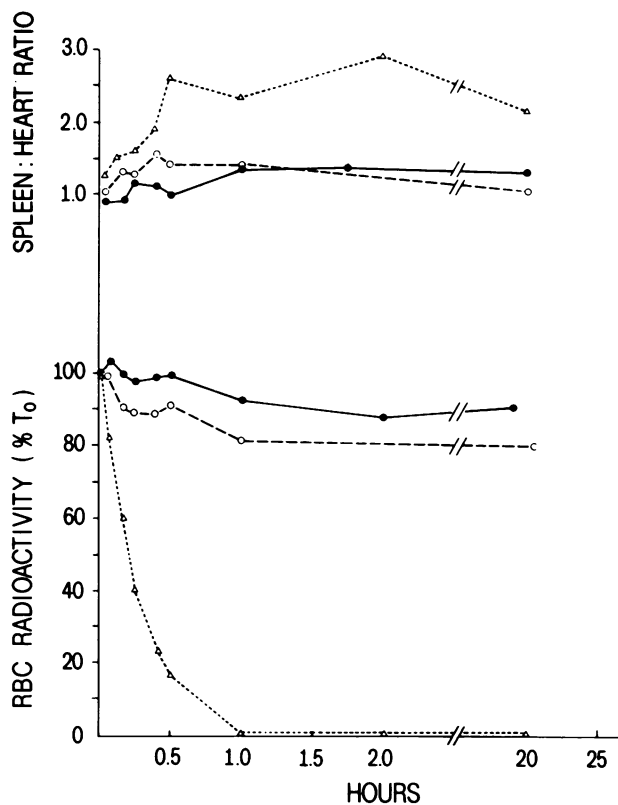


FIGURE 2 (Bottom panel) Dog erythrocyte (RBC) radioactivity, expressed as a percent of initial counts, following reinjection of  $^{51}\text{Cr}$ -labeled cells incubated for 90 min at  $37^\circ\text{C}$ , pH 8 in Tris-buffer (○), 0.2 mM diamide (●), or 0.4 mM diamide (△). (Top panel) Splenic sequestration of  $^{51}\text{Cr}$ , expressed as the ratio of spleen counts to heart counts, observed during the study depicted in the bottom panel. The symbol key is the same as in the bottom panel.

out altering osmotic fragility or forming Heinz bodies (3), and decreased membrane shear elasticity (4). The current investigation revealed similar effects of diamide on dog erythrocytes. Incubation with 0.4 mM diamide markedly decreased GSH, but it did not alter dog erythrocyte ATP. In addition, membrane polypeptide aggregates of two ranges (440,000 and  $>50,000,000$  daltons) formed. We also observed the same type of aggregates in the membranes of human erythrocytes incubated with diamide.<sup>2</sup> These aggregates were similar to those observed in patients with G-6-PD variants accompanied by chronic hemolysis (1, 2), although those found in diamide-incubated cells were more abundant and the spectrin was less condensed into discrete high molecular weight aggregates and dimers. Micropipette deformability was similarly decreased in dog diamide-incubated cells as in human G-6-PD deficient cells (2). Incubation of 0.4 mM diamide-incubated dog erythrocytes with 4 mM dithiothreitol reversed the membrane polypeptide ag-

gregates, normalized erythrocyte micropipette deformability, decreased erythrocyte viscosity, decreased in vivo hemolysis, and splenic sequestration following reinjection. Increased membrane specific gravity resulting from incubation with 0.4 mM diamide was slightly decreased by incubation with dithiothreitol. Therefore, diamide-treated dog erythrocytes have biochemical and membrane characteristics similar to those of cells from patients with G-6-PD variants accompanied by chronic hemolysis. The marked decrease in dog erythrocyte survival that followed reinjection of cells incubated with 0.4 mM diamide observed in the present study provides in vivo evidence indicating that oxidant-induced membrane damage is pathophysiologically related to decreased erythrocyte survival. Furthermore, the significant splenic sequestration of diamide-treated cells is consistent with previous experimental observations that localize the site of the sequestration of oxidant-stressed erythrocytes to the spleen (12). The reversal of diamide-induced membrane polypeptide aggregates by dithiothreitol is also similar to our previous observations in human erythrocytes (1, 2). The normalization of erythrocyte micropipette deformability with the prolongation of in vivo survival observed in cells incubated with diamide followed by dithiothreitol offers strong evidence supporting the concept that oxidant-induced erythrocyte membrane damage is the cause of the hemolysis observed in this study.

Although it is possible that the observed rapid decrease in erythrocyte radioactivity after reinjection was due to elution of  $^{51}\text{Cr}$  from hemoglobin as a consequence of diamide incubation, several lines of evidence strongly argue against this interpretation. The small amount of radioactivity found in the plasma after in vitro incubation (10% after 180 min) compared with the marked decrease in erythrocyte radioactivity in vivo (98% 180 min after reinjection), the small amount of radioactivity recovered from the urine after reinjection (2–5%), and the partial restoration of decreased erythrocyte survival after incubation of diamide-incubated cells in dithiothreitol indicate that the observed decrease in erythrocyte radioactivity following reinjection of 0.4 mM diamide-incubated cells was due to hemolysis. The rapid increase in splenic radioactivity indicated that the primary site of hemolysis was the spleen.

In contrast to cells incubated with 0.4 mM diamide, erythrocytes incubated with 0.2 mM diamide demonstrated in vivo survival equal to that of buffer-incubated control cells. Decreased blood radioactivity noted in the cells incubated in buffer or 0.2 mM diamide during the 1st h after injection was most likely due to erythrocyte membrane damage which occurred during incubation at pH 8. Tris buffer may have contributed to membrane injury during incubation (13).

Our observations of the differential effects of 0.2 and 0.4 mM diamide on dog erythrocytes suggest that the membrane lesion induced by diamide is partially dose dependent. Intact erythrocytes incubated with either 0.2 or 0.4 mM diamide had decreased micropipette deformability, but viscosity was decreased only in cells incubated with 0.4 mM diamide. Cells incubated with the lower diamide concentration had normal GSH and lacked membrane polypeptide aggregates. Higher concentrations of diamide depleted intracellular GSH and resulted in oxidant-induced membrane disulfide bond formation. This lesion was represented by the membrane polypeptide aggregates, although they only indicate a more extensive membrane protein abnormality.<sup>2</sup> Other studies performed in our laboratory show that the increase in membrane specific gravity which follows incubation with 0.4 mM diamide is due to adsorption of cytoplasmic proteins to the membrane cytoskeleton.<sup>2</sup> The fact that specific gravity changed only slightly after incubation with dithiothreitol suggests that this membrane lesion is more slowly reversible. Extensive alteration of the erythrocyte membrane proteins presumably was responsible for the increased viscosity and decreased survival observed in vivo. The apparent dependence of diamide-induced hemolysis on GSH depletion suggests that even though diamide may have a direct effect upon membrane deformability (4), oxidant-induced membrane disulfide bond formation is the more important effect of diamide in regard to in vivo hemolysis. The similarity of cells incubated with 0.4 mM diamide to those seen in G-6-PD variants accompanied by chronic hemolysis, combined with the reversibility of membrane abnormalities of both following incubation with dithiothreitol, indicate that diamide-incubated erythrocytes are a useful model of oxidant-induced membrane injury. The observations made in this study support our hypothesis (2) that oxidant-induced erythrocyte membrane injury is the pathophysiologically significant lesion leading to chronic hemolysis in some G-6-PD variants.

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