Role of vitamin E in glutathione-induced oxidant stress: methemoglobin, lipid peroxidation, and hemolysis

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Abstract Red blood cells (RBC) from normal and vitamin E-deficient rats were incubated in a hypertonic solution of reduced glutathione adjusted to pH 8. Methemoglobin formation occurred in intact RBC from both normal and vitamin E-deficient rats. Hemolysis was significantly greater in RBC from vitamin E-deficient rats. Experiments with catalase, superoxide dismutase, and methional showed that H_2O_2 was the primary extracellular source of oxidant stress. Extracellular superoxide and hydroxyl radical were not involved in oxidant stress. Experiments with dimethyl sulfoxide showed that intracellular hydroxyl radical, generated from H_2O_2, was the hemolytic agent. Neither methemoglobin formation nor lipid peroxidation involved hydroxyl radical. Indeed, lipid peroxidation and hemolysis in RBC from vitamin E-deficient rats were concurrent rather than consecutive events. Phase contrast microscopy showed that rigid, crenated RBC with a precipitate around the interior periphery formed during glutathione-induced oxidant stress. The precipitate dissolved slowly as the crenated RBC were converted to smooth ghosts. It appeared that protein precipitates involving mixed disulfide bonds were reduced and solubilized when extracellular glutathione penetrated the ruptured cell. Comparisons between normal RBC and vitamin E-deficient RBC suggest that vitamin E has little effect on the inward diffusion of extracellular H_2O_2. Vitamin E apparently interacts with different oxidant species derived from intracellular H_2O_2 in preventing lipid peroxidation and the sulfhydryl group oxidation leading to hemolysis.

Supplementary key words hydrogen peroxide · superoxide · hydroxyl radical · catalase · superoxide dismutase · oxygen electrode · trace metal catalysis · methional · ethylene · dimethyl sulfoxide · methane · thiobarbituric acid test · phase contrast microscopy

Rose and György (1) first showed that vitamin E, acting as an antioxidant, protected erythrocytes (RBC) from hemolysis induced by oxidant stress. Later investigators found that hemolysis followed lipid peroxidation (2–11) and it was generally assumed that hemolysis was a consequence of lipid peroxidation. Other studies suggested that a causal relationship between lipid peroxidation and hemolysis is not unequivocally established. H_2O_2 causes lipid peroxidation (4, 7, 8, 10), yet H_2O_2 and agents such as dialuric acid that generate H_2O_2 do not have the same effect in the hemolysis of RBC from vitamin E-deficient rats (11, 12). Hemolysis may not even involve lipid peroxidation. For example, it is possible to separate the oxidant and hemolytic properties of a compound such as menadione (6). Furthermore, hemoglobin precipitates and hemolysis occurs but lipid peroxidation is not detected when RBC are incubated with the oxidant compound acetylphenylhydrazine (13).

Reduced glutathione (GSH) is a particularly attractive agent for studies on vitamin E and oxidant stress in RBC. It was one of the compounds initially studied by Rose and György (1) and has been used in recent studies on lipid peroxidation and the lysis of mitochondria (14, 15) and RBC (1, 16). The autoxidation of GSH yields superoxide (O_2^-), hydroxyl radical (·OH), and H_2O_2 (17). GSH does not penetrate the RBC (18) and therefore the generation of the oxidant species is confined to the extracellular space. In addition, the hypertonic, nonpenetrating GSH solute protects the RBC from purely colloid–osmotic hemolysis (6). Finally, comparisons of the various effects of GSH (16) and H_2O_2 (19) on RBC from normal subjects and those with paroxysmal nocturnal hemoglobinuria (PNH) suggest that it may be possible to separate GSH-induced lipid peroxidation from GSH-induced hemolysis.

We have studied GSH-induced oxidant stress in RBC from normal and vitamin E-deficient rats. Hemolysis was used as a criterion of membrane damage and methemoglobin formation was used as a criterion of intracellular oxidant stress. Lipid peroxidation was estimated by the thiobarbituric acid test. Various oxidant species were studied by the addition of catalase,

Abbreviations: RBC, erythrocytes; GSH, reduced glutathione; PNH, paroxysmal nocturnal hemoglobinuria; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide.

superoxide dismutase (SOD), and several ·OH scavengers to the incubation system.

MATERIALS AND METHODS

Isolation of RBC

Male Sprague-Dawley rats were started as weanlings on either Purina laboratory chow or a pelleted tocopherol-deficient diet for rats (ICN Biochemicals, Plainview, NY). Rats were maintained on these diets until a dialuric acid–hemolysis test (5, 20) showed that a vitamin E deficiency was established with the special diet. A vitamin E deficiency was produced in three different groups of weanling rats and these groups are designated A, B, and C in the tables. Blood was collected by cardiac puncture into a syringe coated with heparin. RBC were separated by centrifugation and the buffy layer was removed by aspiration. The RBC were washed three times by suspending them in their plasma volume of 0.15 M NaCl. Washed, packed RBC were used in all experiments.

Incubation of RBC

GSH was freshly dissolved in distilled water to form a 0.64 M stock solution and was adjusted with concentrated NaOH to approximately pH 8. Other reagents were dissolved in 0.15 M NaCl or saline–phosphate buffer at pH 7.4 (20). SOD (Truett Laboratories) and catalase (purified from beef liver and thymol free, 1 unit/mg, Sigma Chemical Co., St. Louis, MO) were freshly prepared in saline or saline–phosphate. One ml of packed RBC was added to 2 ml of saline or 2 ml of saline–phosphate in a 250-ml round-bottom flask. These mixtures contained the enzyme, $CuCl_2$, and/or ethylenediaminetetraacetic acid (EDTA) at concentrations specified in the tables. Methional (Sigma) and/or dimethyl sulfoxide (DMSO) (Mallinckrodt, St. Louis, MO, analytical reagent) were next added to the suspension. This suspension was incubated for 10 min at 37°C. Two ml of the GSH stock solution was then added. The flask was covered with parafilm and shaken at 100 oscillations/min at 37°C in an Eberbach shaker. Aliquots were removed at specified times and analyzed for hemolysis, methemoglobin, and lipid peroxidation.

In experiments designed to detect ethylene formation from methional and methane formation from DMSO, a 100-ml round-bottom flask fitted with a rubber septum was used in place of the 250-ml round-bottom flask. Aliquots were removed through the septum with a syringe and analyzed by gas–liquid chromatography.

Analytical methods

The degree of hemolysis was estimated by removing 1 ml of the RBC suspension from the incubation system. A 0.1-ml aliquot was added to 10 ml of water containing one drop of 1% Triton X-100 (tube 1). The remaining 0.9 ml of the RBC suspension was then centrifuged at 2000 rpm for 10 min. After centrifugation, a 0.1-ml aliquot of the supernatant was added to 10 ml of water containing one drop of 1% Triton X-100 (tube 2). Absorbancies were measured against a water blank at 575 nm using a Beckman Acta II spectrophotometer and these data were used to calculate percent hemolysis.

Methemoglobin was measured only in intact RBC since, in a concentrated GSH solution, methemoglobin from hemolyzed cells is reduced to hemoglobin. A 0.2-ml aliquot of the RBC suspension was added to a solution containing 4 ml of a 0.1 M phosphate buffer (pH 6.8) and 6 ml of 1% Triton X-100. Methemoglobin, which was not reduced by the dilute GSH solution, was determined in the hemolysate by the cyanomethemoglobin procedure (21). The procedure was modified by increasing the amount of 5% $K_3Fe(CN)_6$ from 0.05 ml to 0.3 ml to insure complete hemoglobin oxidation in the appropriate aliquot of the hemolysate.

A modification of the 2-thiobarbituric acid test (6) was used to measure lipid peroxides. A 0.5-ml aliquot of the RBC suspension was mixed with 1.0 ml of 10% trichloroacetic acid and 2.0 ml of 0.6% 2-thiobarbituric acid. The mixture was heated at 50–60°C for 15 min, cooled, and centrifuged. The absorbance of the supernatant was measured at 534 nm in a Beckman Acta II spectrophotometer and the relative amounts of lipid peroxides were expressed in absorbance units, A_{534} nm.

Methane and ethylene were detected in an Aerograph 1700 chromatograph equipped with a flame ionization detector. Six-foot stainless steel columns containing 80/100 mesh Porapak R (Applied Science Laboratories, State College, PA) were used for separations. The column temperature was 65°C. Helium was the carrier gas and the flow rate was 20 ml/min.

Oxygen consumption

A 0.15 M phosphate buffer (pH 8.0) was placed in a reaction chamber containing a Clark electrode and equilibrated until the temperature of the buffer was maintained at 37°C. The oxygen level of this buffer was then adjusted to 100% on the recorder. The monosodium salt of GSH was dissolved in distilled water and the solution was adjusted to pH 8.0 with concentrated NaOH. The GSH solution was

TABLE 1. Effect of glutathione on hemolysis in erythrocytes from normal and vitamin E-deficient rats

Suspension	% Hemolysis[a]		
	Normal		E-Deficient
	1 hr	2 hr	1 hr
RBC[b]	2.9 (2)	1.4 (1)	2.5 (2)
RBC + GSH	17.3 ± 6.5 (5)	18.3 ± 2.8 (3)	59.4 ± 3.5 (5)
RBC + GSH + 0.2 mg/ml SOD	19.8 ± 9.8 (5)	16.5 ± 11.0 (3)	71.2 ± 1.8 (5)
RBC + GSH + 4 mg/ml catalase	2.6 (2)	3.2 (2)	10.6 ± 6.4 (3)

[a] Mean ± SD. Numbers of determinations are in parentheses.
[b] Weanling population A.

added to the buffer and oxygen consumption was recorded. A fresh GSH stock solution was prepared for each series of experiments. In various experiments, EDTA, SOD, SOD boiled for 5–10 min, or $CuCl_2$ was added to the buffer prior to the addition of GSH.

RESULTS AND DISCUSSION

O_2^- in GSH-induced oxidant stress

Studies with SOD have suggested that O_2^- is involved in the membrane damage associated with oxidant stress. Thus SOD protects against GSH-induced lipid peroxidation with inner mitochondrial fragments (15). It also protects a heavy mitochondrial fraction (22) and a microsomal fraction (23) from xanthine oxidase-induced lipid peroxidation. Further, the hemolytic agent phenylhydrazine generates O_2^- (24). Although the protective effect that was first noted for SOD in dialuric acid-induced hemolysis (25) could not be reproduced (11), SOD did potentiate the protective effect of catalase in dialuric acid-induced hemolysis (11).

The autoxidation of thiols generates O_2^-. The rate of O_2^- formation, which is measured by the reduction of nitroblue tetrazolium to formazan, varies directly with thiol concentration and increasing alkaline pH (17). We found that a 0.01 M GSH solution adjusted to pH 8 reduced 4×10^{-4} M nitroblue tetrazolium. The absorbance at 560 nm increased at an initial rate of 0.016 per min due to the accumulation of blue formazan. The rate was inhibited 56% by 0.3 mg/ml SOD. A concentrated GSH solution is, therefore, an appropriate system for studying the role of externally generated O_2^- in oxidant stress.

Hemolysis occurs when RBC are incubated with hypertonic GSH, and the degree of hemolysis is greater in RBC from vitamin E-deficient rats than in those from normal rats (**Table 1**). These data suggest that hemolysis is the result of oxidant stress. SOD has no effect on GSH-induced hemolysis in RBC from normal rats and it actually enhances the hemolytic process in RBC from vitamin E-deficient rats. These data indicate that O_2^- and secondary radicals from O_2^-, generated in the medium, are not directly involved in GSH-induced oxidant stress.

The stimulatory effect (Table 1) of SOD on GSH-induced hemolysis in RBC from vitamin E-deficient rats was unexpected. This observation can be ex-

TABLE 2. Relative rate of oxygen consumption by glutathione solutions at pH 8.0 and 37°C

Solution	Relative Rate of O_2 Consumption
Control (6.9×10^{-2} M GSH)	1.0[a]
Control + 1×10^{-2} M EDTA	0.6 ± 0.2 (3)[b]
Control + 5×10^{-2} M EDTA	<0.1 (3)[c]
Control + 250 μg/ml SOD	1.9 ± 0.4 (15)[b]
5×10^{-2} M EDTA added	<0.1 (2)[c]
Control + 250 μg/ml boiled SOD	>10.0 (6)[c]
5×10^{-2} M EDTA added	<0.1 (2)[c]
Control + 1.55×10^{-5} M $CuCl_2$	>10.0 (6)[c]
1×10^{-2} M EDTA added	1.2 (1)
5×10^{-2} M EDTA added	0.4 (1)

[a] Absolute rate of oxygen consumption was 6.0 ± 3.2% per min. (mean ± SD for 43 determinations).
[b] Mean ± SD. Numbers of determinations are in parentheses.
[c] Rate of oxygen consumption was too low (<0.1) or too high (>10) for accurate measurement.

TABLE 3. Effect of glutathione on methemoglobin formation by erythrocytes from normal rats

Suspension	% Methemoglobin[a]	
	1 hr	2 hr
RBC	0 (2)	1.4 (1)
RBC + GSH	1.6 ± 1.3 (9)	3.1 ± 2.5 (5)
RBC + GSH + 0.2 mg/ml SOD	17.2 ± 5.7 (7)	46.5 ± 14.8 (5)
4 mg/ml catalase added	2.5 ± 0.7 (4)	2.6 ± 0.8 (3)
RBC + GSH + 1.2×10^{-5} M $CuCl_2$	66.5 ± 20.4 (3)	91.0 ± 15.6 (3)
4 mg/ml catalase added	4.6 (2)	2.7 (1)

[a] Mean ± SD. Numbers of determinations are in parentheses.

plained by the catalytic effect of a trace metal loosely bound to the SOD. Trace metals are required for measurable thiol oxidation. Thus EDTA in the appropriate concentration abolished oxygen consumption in a GSH solution (**Table 2**). SOD enhances the rate of oxygen consumption; this SOD effect is abolished by EDTA (Table 2), which will complex with loosely bound, but not tightly bound, metal ions in the active site of the SOD enzyme (26). Boiled SOD profoundly enhances the rate of oxygen consumption and this effect is also abolished by EDTA (Table 2). The Cu^{2+} ion, in the same concentration as that which could be liberated from denatured 7.7×10^{-6} M SOD containing two atoms of Cu^{2+} per molecule in the active site (26), greatly enhances the rate of oxygen consumption; this Cu^{2+} effect is also abolished by EDTA (Table 2). These data show that a trace metal in the SOD preparation increases oxidant stress by catalyzing thiol autoxidation.

In RBC exposed to oxidant stress, methemoglobin is formed after intracellular GSH is depleted (27). Any methemoglobin released by hemolysis into the hypertonic GSH solution will be reduced to hemoglobin. Methemoglobin is a true measure of intracellular oxidant stress induced by extracellular GSH because it will be contained by intact RBC only.

In RBC from normal rats, SOD does not enhance hemolysis (Table 1); however, it does have a profound effect on methemoglobin production. GSH-induced oxidant stress is itself insufficient to form significant amounts of methemoglobin, even in a 2-hr incubation period (**Table 3**), but the addition of SOD or $CuCl_2$ augments oxidant stress and leads to the formation of increasing amounts of methemoglobin throughout the 2-hr incubation period (Table 3). These data support the concept that trace metal catalysis enhances oxidant stress. Furthermore, the comparison of RBC from normal and vitamin E-deficient rats shows that vitamin E protects the membrane from hemolysis (Table 1) but does not protect intracellular hemoglobin from oxidation (Table 3). Thus vitamin E does not function as a nonspecific membrane barrier to oxidant stress.

H_2O_2 in GSH-induced oxidant stress

Rose and György (1) first demonstrated with catalase that oxidant stress with dialuric acid involved H_2O_2. Subsequent investigators found that hemolysis in primaquine-sensitive individuals involved the generation of H_2O_2 by an oxidant drug (28). H_2O_2 is formed during the autoxidation of thiols (17) and intracellular H_2O_2 accumulates when RBC are incubated aerobically with hypertonic GSH (16). These studies suggest that GSH-induced oxidant stress involves the extracellular generation of H_2O_2 which then acts as the oxidant species.

Catalase protects RBC of both vitamin E-deficient and normal rats from GSH-induced hemolysis (Table 1). It prevents methemoglobin formation in RBC from normal rats even when GSH oxidation is catalyzed by trace metals in SOD or $CuCl_2$ (Table 3). The protective effect of catalase is apparently related to its enzyme activity since heat denaturation at 70°C partially eliminates the protective effect of 4 mg/ml catalase and almost completely eliminates the protective effect of 1 mg/ml catalase (**Table 4**). Thus, catalase data (Tables 1, 3, 4) are consistent with the concept that H_2O_2, generated in the GSH solution, is the extracellular source of oxidant stress both for hemolysis and for methemoglobin formation.

Intracellular ·OH and GSH-induced oxidant stress

Studies with catalase (Tables 1, 3, and 4) identify H_2O_2 as the extracellular oxidant in GSH-induced oxidant stress. These studies do not distinguish between H_2O_2 and a radical formed from H_2O_2, such as ·OH (28), as the immediate source of membrane damage and hemoglobin oxidation. Furthermore, the data do not distinguish between the extracellular and/or intracellular surfaces of the RBC as the site(s) of membrane damage. We used GSH (29), methional (30–32), and DMSO (29, 32–34) as ·OH scavengers to investigate these questions.

The presence of free radicals in a solution is demonstrated by the formation of ethylene from methional (31), and ethylene is generated when methional is added to a GSH solution (**Fig. 1**). It is unlikely, however, that the extracellular free radical ·OH is involved in hemolysis. GSH is itself an effective trapping agent for ·OH (29) and ·OH will not persist as the radical species in a concentrated GSH solution.

TABLE 4. Role of concentration and denaturation on the protective effect of catalase during glutathione-induced hemolysis in vitamin E-deficient rats

Suspension	% Hemolysis[a] 30 min		60 min	
RBC[b]	1.1	(2)	1.2	(2)
RBC + GSH	48.1 ± 2.8	(4)	66.8 ± 5.5	(4)
RBC + GSH + 4 mg/ml catalase	4.7	(2)	7.7	(2)
RBC + GSH + 4 mg/ml catalase (70°C)[c]	27.6	(1)	31.0	(1)
RBC + GSH + 1 mg/ml catalase	8.3	(1)	28.6	(1)
RBC + GSH + 1 mg/ml catalase (70°C)[c]	30.8	(1)	60.5	(1)

[a] Mean ± SD. Numbers of determinations are in parentheses.
[b] Weanling population C.
[c] The catalase solution was heated at 70°C for about 15 min before addition to RBC.

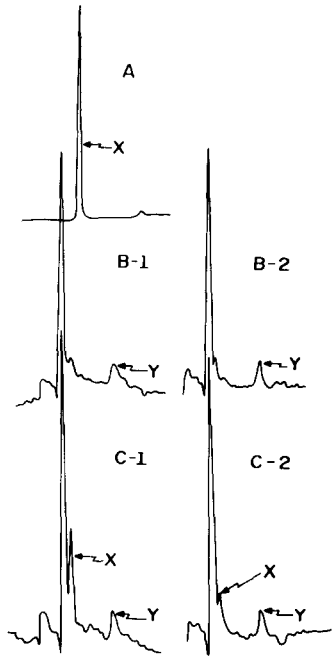

Fig. 1. GLC tracings for methane (*X*) and ethylene (*Y*) formation during GSH-induced hemolysis. The tracing for natural gas is shown in (*A*) to identify methane. Tracings for RBC and GSH incubated with 0.7 M DMSO and 0.009 M methional show an ethylene peak at 30 min (*B-1*) and a pronounced methane peak at 2 hr (*C-1*). Tracings for GSH alone incubated with 0.7 M DMSO and 0.009 M methional show an ethylene peak at 30 min (*B-2*) and the absence of a pronounced methane peak at 2 hr (*C-2*).

Furthermore, the highly effective free radical trapping agent methional (30–32) does not protect vitamin E-deficient RBC from GSH-induced hemolysis (**Table 5**) even though it traps free radicals with the formation of ethylene (Fig. 1). These data support the hypothesis that H_2O_2 is a necessary extracellular agent for hemolysis.

DMSO is both an ·OH-trapping agent (29, 32–34) and a rapidly penetrating solute (35) and it inhibits hemolysis in RBC from both normal and vitamin E-deficient animals (Table 5). Several aspects of the DMSO protective effect show that DMSO acts to alleviate oxidant stress. Thus normal cells, which already contain the antioxidant vitamin E, are protected more effectively by 0.7 M DMSO than are vitamin E-deficient cells ($P < 0.001$), while both normal and vitamin E-deficient cells are protected to the same extent by 1.3 M DMSO. It is interesting that the extracellular radical scavenger, methional, potentiates ($P < 0.01$ at 30 min and $P < 0.05$ at 60 min) the protective effect of 0.7 M DMSO with RBC from vitamin-E deficient rats (Table 5).

DMSO may protect against oxidant stress by serving as an ·OH trapping agent; alternatively, it may allow the penetration of extracellular GSH into the RBC, a process that does not occur under normal conditions (18). GSH from the medium could then act as a substrate for RBC glutathione peroxidase (27) in protecting the cell from oxidant stress. Several studies show that DMSO does not affect GSH transport into the RBC. In one experiment, a series of GSH solutions containing tritiated GSH (New England Nuclear Corp., Boston, MA) and RBC from normal rats were incubated for 30 or 60 min either alone or with 0.7 M or 1.3 M DMSO added. In all cases the RBC were washed four times. Since labeled GSH is bound to the RBC membrane (16), trichloroacetic acid was used to lyse the RBC and precipitate protein. When the supernatant, which would contain intracellular GSH and its oxidation product, GSSG, was examined, it was shown to have only traces of radioactivity when DMSO had been included in the incubation medium. The supernatant contained much more radioactivity when DMSO had been omitted. In the latter case, however, the RBC clumped and were difficult to resuspend after centrifugation. The increase in supernatant radioactivity probably reflects the difficulty encountered in washing and resuspending RBC in the absence of DMSO.

TABLE 5. Effect of hydroxyl radical scavengers on glutathione-induced hemolysis in RBC from normal and vitamin E-deficient rats

	% Hemolysis[a]			
	Normal		E-Deficient	
Suspension	30 min	60 min	30 min	60 min
RBC[b] + GSH	21.7 ± 7.0 (13)	32.0 ± 6.0 (13)	34.7 ± 5.0 (13)	45.0 ± 6.6 (19)
RBC + GSH + 0.009 M methional			35.9 (2)	37.3 ± 1.7 (3)
RBC + GSH + 0.7 M DMSO	5.0 ± 0.9 (3)	9.4 ± 1.0 (3)	20.7 ± 7.8 (6)	27.6 ± 11.5 (6)
RBC + GSH + DMSO-methional[c]			7.7 ± 3.8 (5)	13.3 ± 5.5 (5)
RBC + GSH + 1.3 M DMSO	4.4 ± 1.2 (4)	9.4 ± 2.0 (4)	5.0 ± 1.3 (8)	8.8 ± 2.7 (9)

[a] Mean ± SD. Numbers of determinations are in parentheses.
[b] Weanling population B.
[c] Suspension contained 0.7 M DMSO and 0.009 M methional.

TABLE 6. Effect of DMSO on glutathione-induced hemolysis and methemoglobin formation in erythrocytes from normal rats

Suspension	% Hemolysis	% Methemoglobin
	1 hr	
RBC[a] + GSH	32.0 ± 6.0 (13)[b]	1.2 ± 1.4 (4)[c]
RBC + GSH + 0.2 mg/ml SOD	38.4 ± 17.6 (5)	23.5 ± 1.9 (5)
RBC + GSH + 0.2 mg/ml SOD + 1.3 M DMSO	5.1 ± 1.0 (5)	33.8 ± 5.9 (5)

[a] Weanling population B.
[b] Mean ± SD for percent hemolysis. Numbers of determinations are in parentheses.
[c] Mean ± SD for percent methemoglobin in unhemolyzed RBC fraction. Numbers of determinations are in parentheses.

A second experiment involved DMSO and methemoglobin formation in response to GSH-induced oxidant stress. Since glutathione peroxidase protects cells from oxidant stress, little methemoglobin is formed until intracellular GSH is depleted (18, 27, 28). DMSO should decrease, or even prevent, methemoglobin formation if it makes the RBC permeable to extracellular GSH. In RBC from our second control animal population, we found that SOD again catalyzed the formation of methemoglobin in response to oxidant stress induced by GSH (**Table 6**). DMSO protected these RBC against hemolysis (Table 6); however, more methemoglobin was formed in intact RBC when DMSO was included in the medium than was formed when it was omitted (Table 6). These data show that DMSO stabilizes GSH-depleted RBC without enhancing their GSH permeability.

Ashwood-Smith (33) has noted that the protective effect of DMSO against ·OH is afforded by the combination of ·OH with DMSO and the subsequent formation of CH_3·. The CH_3· radical may then abstract H· to form methane. Very little methane is formed when DMSO is added to a GSH solution (Fig. 1). However, a significant amount of methane is produced in 2 hr (Fig. 1) when RBC in a GSH solution are protected against hemolysis by the addition of DMSO. (Hemolysis data at 2 hr for the incubations described in Fig. 1 are 23.4% in RBC incubated with DMSO and methional, and 61% in RBC incubated without the protecting agents. RBC used in these experiments were obtained from weanling population C.)

The DMSO data strongly suggest that ·OH, formed from the H_2O_2 that accumulates within the RBC, is responsible for the membrane damage leading to hemolysis. Since DMSO protects the RBC against hemolysis but has no effect on methemoglobin formation, it is further apparent that ·OH is not involved directly in hemoglobin oxidation.

Lipid peroxidation and hemolysis

Many studies relate lipid peroxidation in vitamin E deficiency to hemolysis (1–12). Lipid peroxidation may involve ·OH (36), and our studies with DMSO and methional show that intracellular ·OH is formed during GSH-induced oxidant stress. Lipid peroxidation alters the permeability of synthetic lipid bilayers, an effect prevented by vitamin E (37). Thus GSH-induced hemolysis in RBC from vitamin E-deficient rats could be the direct consequence of lipid peroxidation from intracellular ·OH.

Lipid peroxidation and hemolysis both increase when RBC from vitamin E-deficient rats are subjected to GSH-induced oxidant stress (**Table 7**). The ·OH scavenger, DMSO, inhibits hemolysis without inhibiting lipid peroxidation (Table 7); indeed, lipid peroxidation at 60 min is enhanced a small but significant amount ($P < 0.05$) by its presence. These data suggest that ·OH is not involved directly in lipid peroxidation. Furthermore, they show that hemolysis is not the direct consequence of lipid peroxidation. Thus vitamin E inhibits both lipid peroxidation and hemolysis.

Morphology of RBC during GSH-induced oxidant stress

RBC from vitamin E-deficient rats were incubated in a GSH solution and examined by phase contrast microscopy at 10, 30, and 50 min (**Fig. 2**). The cells were crenated in the hypertonic GSH medium. Crenated RBC may not be significant in themselves, since slight crenation was also seen when RBC were incubated with saline–phosphate buffer alone, but the crenated cells in the GSH incubation were rigid and contained a precipitate, presumably denatured hemoglobin and methemoglobin, that was concentrated at the periphery (Fig. 2-B). These precipitates did not resemble Heinz bodies in phase contrast microscopy, and Heinz bodies were not

TABLE 7. Effect of DMSO on glutathione-induced hemolysis and lipid peroxidation in erythrocytes from vitamin E-deficient rats

Suspension and Incubation Time	% Hemolysis[a]	TBA (A_{534} nm)[a]
RBC[b] + GSH		
30 min	31.3 ± 0.4 (5)	0.092 ± 0.021 (4)
60 min	49.6 ± 4.0 (5)	0.160 ± 0.036 (4)
120 min	58.6 ± 6.6 (5)	0.212 ± 0.057 (5)
RBC[b] + GSH + 1.3 M DMSO		
30 min	5.1 ± 1.2 (5)	0.118 ± 0.022 (4)
60 min	9.7 ± 2.9 (5)	0.218 ± 0.023 (4)
120 min	12.5 ± 4.0 (5)	0.244 ± 0.045 (5)

[a] Mean ± SD. Numbers of determinations are in parentheses.
[b] Weanling population B.

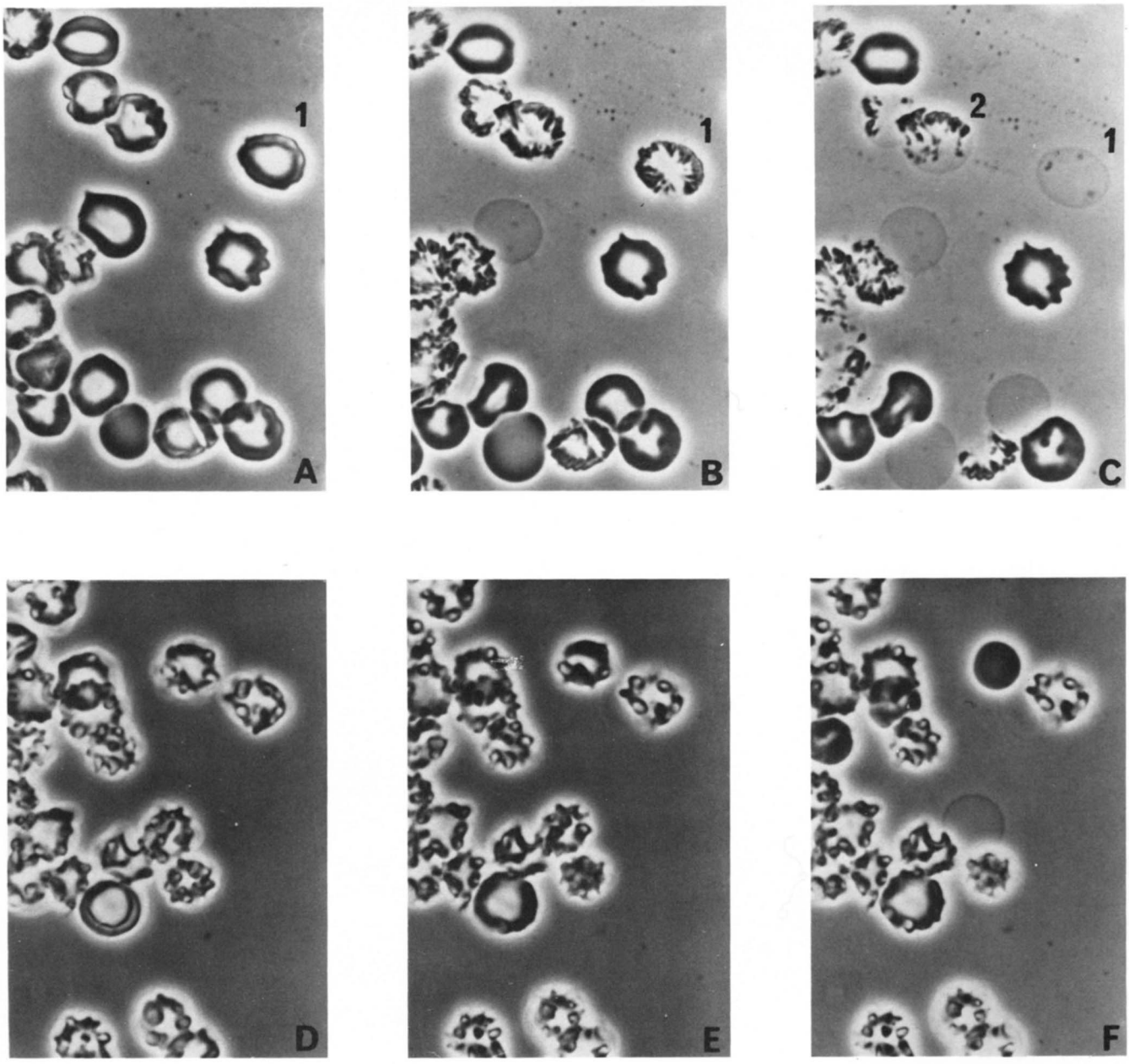

Fig. 2. Phase contrast microscopy of vitamin E-deficient RBC. *A*, *B*, and *C* show RBC incubated for approximately 10, 30, and 50 min in hypertonic GSH without DMSO. In cell *1*, note the transformation from crenated cell (*A*) to a precipitate at the periphery (*B*) and finally to a smooth ghost (*C*). In cell *2*, note the presence of both smooth percipitate-free and crenated precipitate-containing peripheral segments (*C*). *D*, *E*, and *F* show RBC incubated for approximately 10, 30, and 50 min in hypertonic GSH containing 1.3 M DMSO. Note the absence of precipitate and that relatively little lysis occurs.

detected when the RBC were stained with crystal violet. The RBC tended to lose their irregular shape as they were converted to hemoglobin-free ghosts (Fig. 2-C). This process occurred over a long time interval and the same cell often showed a smooth precipitate-free membrane segment as well as a crenated precipitate-containing membrane segment (Fig. 2-C). Thus GSH-induced hemolysis differed from colloid–osmotic hemolysis where hemoglobin-free ghosts are formed almost instantaneously at the time of hemolysis.

When DMSO was added to the hypertonic GSH medium (Fig. 1-D), the crenated RBC resembled echinocytes (38). No precipitate was observed, even

when the cells were incubated for a longer time (Fig. 1-E, 1-F), and rounded cells that still contained their heme pigments were observed at 50 min (Fig. 1-F).

RBC morphology shows that oxidant stress, which is known to alter membrane sulfhydryl groups (4), leads to the formation of rigid and irregularly shaped cells containing denatured hemoglobin that appears to be bound to the membrane. The cells rupture and are slowly converted to hemoglobin-free ghosts. We suggest that the rigid cells and denatured hemoglobin are caused by the formation of mixed disulfide bonds between membrane elements and between hemoglobin and membrane elements. These disulfide bonds are reduced by extracellular GSH after the cells rupture. Other investigators have reported that Heinz bodies are formed from mixed disulfide bonds between hemoglobin and the cell membrane (39), but we did not observe them in our studies. Recently Sears, Friedman, and White (40) showed that Heinz bodies did not involve mixed disulfide bonds, so our hypothesis is consistent with an absence of observable Heinz bodies. It appears that Heinz bodies may be formed from oxidant stress on hemoglobin alone, while the precipitate that we observed is formed from oxidant stress on both the hemoglobin and the cell membrane. Thus, when the membrane is protected with DMSO (Fig. 2), a precipitate is not formed even though hemoglobin is oxidized to methemoglobin in the presence of DMSO (Table 6).

Suggested roles for vitamin E in lipid peroxidation and hemolysis

GSH-induced oxidant stress leads to intracellular methemoglobin formation, lipid peroxidation, and hemolysis. Studies with catalase show that the primary source of the oxidant stress is extracellular H_2O_2. The H_2O_2 diffuses through the cell membrane of the RBC without damaging the membrane just as it diffuses through the cell membrane of the granulocyte (41) without causing damage. Studies with $\cdot OH$ scavengers show that intracellular H_2O_2 generates $\cdot OH$ which damages the cell membrane and so leads to hemolysis, a process that may be related to the role of $\cdot OH$ in phagocytosis (42, 43). These studies also show that $\cdot OH$ is not involved in lipid peroxidation. Thus lipid peroxidation occurs even when hemolysis is prevented by $\cdot OH$ scavengers. Since lipid peroxidation probably involves $^1O_2$ (oxygen singlet) (22, 44–46), these observations suggest that GSH-induced oxidant stress requires the intracellular decomposition of H_2O_2 both to $\cdot OH$ for hemolysis and to $^1O_2$ for lipid peroxidation.

The RBC is well adapted for the formation of $\cdot OH$ and $^1O_2$ from H_2O_2. Intracellular O_2^- is formed during the autoxidation of hemoglobin (47–49) and is probably involved in oxidative hemolysis (50). The oxidant species, $\cdot OH$ and $^1O_2$, are both formed when O_2^- reacts with H_2O_2 (22, 44, 49, 51); indeed, this reaction may occur at the interior surface of the membrane as extracellular H_2O_2 diffuses through the membrane and mixes with intracellular O_2^-. Misra and Fridovich (52), in explaining the oxygen enhancement of radiation lethality, recently suggested a similar reaction between O_2^- and H_2O_2 at the surface of a bacterium.

Although our studies strongly suggest that intracellular $\cdot OH$ is the hemolytic agent, they do not eliminate the possibility that an extracellular radical derived from the oxidizing species may have a cooperative effect on hemolysis. A cooperative effect could explain why H_2O_2 is less effective than dialuric acid as hemolytic agent (11, 12). The formation of ethylene from methional (Fig. 1) shows that free radicals are formed in the GSH solution. The methional–DMSO mixture may protect against hemolysis (Table 5) by partially scavenging extracellular (methional) and intracellular (DMSO) free radicals.

Lipid peroxidation and hemolysis, which appeared in our studies as concurrent rather than consecutive events, are both inhibited by vitamin E. Thus vitamin E protects lipids from oxidation, probably by $^1O_2$, and protects sulfhydryl groups from oxidation by $\cdot OH$. It is difficult to imagine how the sulfhydryl groups are protected by vitamin E that is dissolved or randomly dispersed in the membrane lipid. Recent studies (53) suggest that vitamin E assumes a specific orientation in the RBC membrane and that this orientation could explain its protective effect on membrane sulfhydryl groups.

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