

# Spectrofluorescent Detection of In Vivo Red Cell Lipid Peroxidation in Patients Treated with Diaminodiphenylsulfone

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**ABSTRACT** In the absence of vitamin E deficiency, red cell lipid peroxidation has not been clearly demonstrated in freshly drawn blood obtained from patients with various hemolytic anemias despite indirect evidence that oxidative decomposition of cell membrane unsaturated fatty acids occurs in these particular hemolytic states. Recent studies have indicated that malonaldehyde, a decomposition product of oxidized polyunsaturated fatty acids, is able to covalently cross-link the amino groups of protein or lipid resulting in a fluorescent compound. In the present study we have utilized spectrofluorescent technique to assess whether such fluorescence is present in red cell lipid extracts in association with lipid peroxidation.

In vitro red cell lipid peroxidation produced by ultraviolet radiation or the oxidant gas ozone was associated with the development of a fluorescent peak (excitation maximum 360 nm; emission maximum 440 nm) in lipid-containing red cell extracts. Similar fluorescence was observed after incubation of red cells with malonaldehyde or with malonaldehyde-containing extracts of peroxidized red cell lipid. Spectrofluorescent evaluation of chloroform:isopropanol extracts obtained from the freshly drawn red cells of six patients receiving the oxidant hemolytic drug diaminodiphenylsulfone also revealed a peak at 440 nm which ranged from 39 to 78 U. In contrast, the levels in samples obtained from 11 hematologically normal subjects were 17–27 fluorescence U. No evidence for an increase in blood levels of free mal-

naldehyde was observed using the 2-thiobarbituric acid test which is the most commonly performed assay of lipid peroxidation. Serum vitamin E levels were within the normal range. Density separation indicated that the bulk of the fluorescence was present in older red cells. A similar fluorescent peak was also observed in lipid-containing extracts of red cells obtained from rabbits repeatedly injected with phenylhydrazine.

The finding of fluorescent spectra consistent with the cross-linking of aminolipid by malonaldehyde in the red cells of patients receiving diaminodiphenylsulfone indicates that in vivo red cell lipid peroxidation does occur in the absence of vitamin E deficiency.

## INTRODUCTION

The peroxidation of red cell membrane unsaturated fatty acids has been suggested to contribute to hemolysis in a number of entities including vitamin E-deficient states, paroxysmal nocturnal hemoglobinuria, thalassemia, and treatment with hyperbaric oxygen or oxidizing drugs (1–12). Detection of red cell lipid peroxidation has for the most part depended on the 2-thiobarbituric acid (TBA)<sup>1</sup> test which predominantly measures malonaldehyde (MDA), a three-carbon dialdehyde formed during the peroxidative decomposition of polyunsaturated fatty acids. It is of note that the assumption of in vivo human red cell lipid peroxidation has usually been inferred from the in vitro demonstration of TBA reactants during the incubation of red cells in situations thought to mimic the clinical condition. With the exception of vitamin E-deficient neonates (3), the TBA test has

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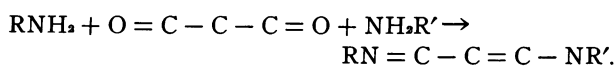
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<sup>1</sup> Abbreviations used in this paper: C:I, chloroform:isopropanol; DDS, diaminodiphenylsulfone; MDA, malonaldehyde; TBA, thiobarbituric acid.

consistently failed to demonstrate free MDA in freshly drawn human blood. This is, presumably, partially due to the reactivity of the carbonyl groups of MDA which results in the formation of complexes no longer detectable in the TBA test.

MDA, as well as other carbonyl compounds, is able to covalently link to free amino groups by forming Schiff bases. Recently, Tappel and his associates have observed that when both carbonyl groups of MDA reacted with protein or phospholipid amino groups, a fluorescent compound resulted (13-16). The general reaction leading to the production of a fluorescent amino-iminopropene derivative is as follows:



Fluorescent spectra consistent with the presence of this derivative have been observed in extracts of vitamin E-deficient tissue and of microsomal suspensions undergoing lipid peroxidation (17, 18).

In the present study we demonstrate that fluorescence consistent with the cross-linking of MDA to aminolipid occurs in association with *in vitro* red cell lipid peroxidation, and that similar fluorescence is present in lipid-containing extracts of red cells obtained from patients receiving the oxidant hemolytic drug diaminodiphenylsulfone (DDS).

## METHODS

Blood, anticoagulated with EDTA (1.2 mg/ml), was obtained from patients with dermatitis herpetiformis, from hematologically normal volunteers, and from patients with reticulocytoses due to autoimmune hemolytic anemia or response to hematinics. No difference in fluorescence was observed when EDTA-anticoagulated blood was compared with blood defibrinated by means of glass beads. The blood was immediately washed three times in 0.01 M phosphate-buffered saline, pH 7.4, with removal of the buffy coat and resuspended in this buffer to a hematocrit of 80%. Lipid extracts were obtained on duplicate 1-ml samples. Blood obtained by cardiac puncture from New Zealand white rabbits was tested similarly. The entire procedure, including fluorescence determination, was performed as rapidly as possible and completed on the day the blood was drawn. Red cell glutathione was measured by the method of Beutler et al. (19), and methemoglobin was assessed by the method of Hegesh et al. (20).

For incubation studies, normal human blood was washed three times in 310 mosmol phosphate buffer, pH 7.0, and resuspended in this buffer to the appropriate hematocrit for individual studies. MDA was prepared from MDA bis-(dimethylacetal) by shaking with Dowex 50 (Dow Chemical Co., Midland, Mich.) (21) and stored for up to 3 days in the dark at 4°C. MDA-containing saline extracts of peroxidized lipid were obtained by the procedure of O'Malley et al. (22). Red cell suspensions were incubated in MDA at 25°C for up to 24 h. Reagent-grade toluene was added to retard microbial growth when incubation periods extended overnight. This did not affect fluorescence. Lipid peroxidation was produced either by exposing red cells or ghost sus-

pensions in fritted disk bubblers (Ace Glass, Inc., Vineland, N. J.) to 20 ppm. ozone as previously described (23) or by irradiating these suspensions with ultraviolet light utilizing a 450-W mercury lamp (Ace-Hanovia photochemical reactor, Ace Glass, Inc.). The levels of ozone or ultraviolet irradiation utilized did not directly produce hemolysis. The TBA test for lipid peroxidation was performed as previously described (24).

Red cell and ghost lipid was extracted in duplicate using carbonyl-free fluorescent-grade chloroform and isopropanol according to the procedure of Rose and Oklander (25). Where possible, the chloroform:isopropanol (C:I) extraction steps were performed under nitrogen to prevent artifactual lipid peroxidation. The final extract was filtered through glass wool, and the fluorescence was evaluated immediately thereafter. Fluorescence excitation and emission spectra were obtained at 25°C on a Hitachi-Perkin Elmer MPF 3 spectrofluorometer (Perkin-Elmer Corp., Mountain View, Calif.) in the direct mode standardized with quinine sulfate. The measured wavelength was not corrected for internal instrument distortion. Excitation and emission slits were set at 8 nm, and the sensitivity was set at "30". Fluorescence was recorded as the peak height at 440 nm and expressed in arbitrary units based on these instrument settings. The fluorescence levels of duplicate C:I extractions were generally within 10% of each other. Fluorescamine-reactive amino groups were measured by a minor modification of the procedure of Udenfriend et al. (26), and lipid phosphorus was measured by the method of Bartlett (27).

Chloroform and isopropanol was found to be preferable for extraction of red cells to the method previously described by Tappel et al. (17). These investigators have utilized a modified Folch procedure in which amino-iminopropene fluorescence in the nonaqueous phase is ascribed to MDA cross-links of aminolipid, predominantly the free amino groups of phosphatidyl ethanolamine and phosphatidyl serine, while similar fluorescence in the aqueous phase is believed to be due to MDA cross-links of protein amino groups. However, in this procedure, hemoglobin or its derivatives are present in variable amounts in the nonaqueous phase as well as being a major component of the aqueous phase. This hemoglobin contamination, which is not observed in C:I lipid extracts, presents two experimental problems: the absorption peaks of hemoglobin and its derivatives overlap the fluorescence emission of amino-iminopropene derivatives and thus complicate interpretation of the observed fluorescent spectra, and heme is an excellent potentiator of lipid peroxidation, thereby increasing the possibility of artifactual lipid peroxidation during the preparatory procedure. Similarly, attempts to assess the presence of red cell membrane protein cross-links in patients treated with DDS or in rabbits receiving phenylhydrazine by first preparing red cell ghosts and then evaluating the aqueous phase of a Folch extract, were complicated by the inability to prepare hemoglobin-free ghosts in these instances, presumably due to the presence of Heinz bodies. These technical difficulties complicate determination of whether fluorescent protein cross-links occur in the red cell membranes of DDS-treated patients but do not preclude their presence.

## RESULTS

The fluorescence excitation and emission spectra of C:I extracts of red cells exposed to 20 ppm. ozone for 2 h are shown in Fig. 1. A fluorescent peak is present with an excitation maximum at 360 nm and an emission maxi-

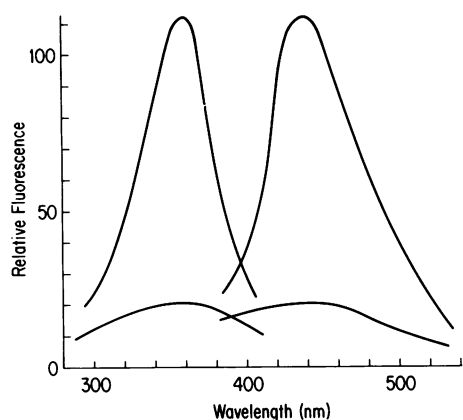


FIGURE 1 Fluorescence excitation and emission spectra of chloroform:isopropanol extracts of red cells previously exposed to 20 ppm. ozone for 2 h demonstrating an excitation maximum at 360 nm and an emission maximum at 440 nm. The lower curves were obtained from similar extracts of control red cells and show no distinct peaks.

mum at 440 nm. A similar peak was observed after ultraviolet irradiation. The 360-nm excitation spectrum of C:I extracts obtained from unozonized red cell suspensions show a slight shoulder emission at 440 nm. The presence of this fluorescence in the control red cell samples was unaffected by further antioxidant precautions including performing the extraction procedure in a glove bag under nitrogen and the addition of 1 mg/ml butylated hydroxytoluene to the solvents.

Incubation of normal human red cells with two levels of MDA or with MDA-containing saline extracts of ozonized or ultraviolet-irradiated red cell lipid also resulted in the formation of a fluorescent peak at 440 nm (Table I). The levels of TBA-reacting material before

TABLE I  
Fluorescence\* of C:I Extracts after Incubation of Red Cells with TBA Reactant-Containing Solutions

Incubation medium	TBA reactants at start of incubation	Fluorescence
	nmol MDA / ml RBC†	
Saline extract		
Control RBC lipid	2	26
Ozone-exposed RBC lipid	46	270
Ultraviolet-irradiated RBC lipid	58	315
Control	0	21
Malonaldehyde	67	260
Malonaldehyde	667	3,100

Incubations were performed in duplicate at 25°C for 16 h.

\* Fluorescence, 360 nm excitation; 440 nm emission.

† RBC, red blood cells.

incubation, also shown in this table, are approximately proportional to the intensity of the resultant fluorescence.

To compare this fluorescence assay with the TBA test, both measurements were performed in normal human red cell ghosts (0.08 mg protein/ml) gassed with 1 ppm. ozone for 4 h (Fig. 2). At each time period, two aliquots of the ghost sample were removed from ozone exposure, in one of which TBA reactants and the fluorescence of C:I extracts were measured immediately (depicted by the solid lines) while the other aliquot was allowed to stand overnight before assay of these parameters (shown as a dotted line). The increase in TBA reactants is more apparent at the onset of ozone exposure and tends to rise to maximal levels after 3-4 h. In contradistinction, fluorescence only gradually increases during the initial period, but rises as ozone exposure continues. Furthermore, in those samples allowed to stand overnight before assay, an increase in fluorescence is observed while there is a loss in TBA reactants, presumably reflecting the covalent linkage of free MDA to membrane constituents. In control samples gassed with charcoal-filtered room air and incubated in parallel with the ozone-exposed ghosts, only minimal increases in TBA reactants (<6 nmol MDA/mg protein) and fluorescence (<27 U) were observed.

*Patient studies.* The 440-nm fluorescence emission of C:I extracts of red cells obtained from six patients receiving DDS in the dose range of 100-200 mg daily ranged from 39 to 78 U (Fig. 3). In comparison, the levels in samples obtained from 11 hematologically nor-

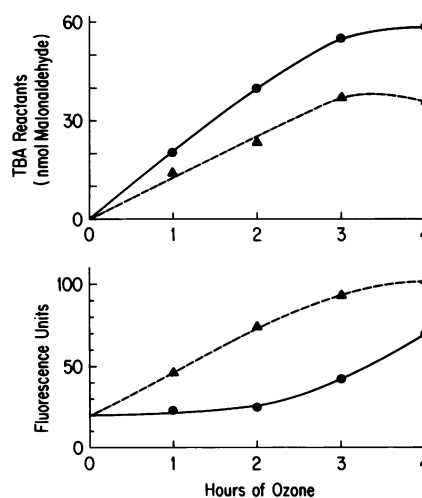


FIGURE 2 TBA reactants in ghost suspensions (0.08 mg protein/ml) gassed with 1 ppm. ozone and the 440-nm fluorescence (360 nm excitation) of C:I extracts of the same ghost samples. At each time period, two aliquots were removed from ozone exposure, one of which was tested immediately (—) while the other was allowed to stand at 25°C overnight (----) before assay.

mal subjects were 17–27 U, which was similar to the findings in patients with reticulocytoses due to autoimmune hemolytic anemia or response to hematinics (15–25 U fluorescence). This difference between the DDS-treated patients and either control group is statistically significant at  $P < 0.01$  (Student's  $t$  test).

One patient with dermatitis herpetiformis was followed from the inception of DDS therapy, initially with 100 mg daily for 3 days and then with 200 mg daily, until he was lost to follow-up. As shown in Fig. 4, there was a gradual increase in fluorescence associated with a reticulocytosis, the presence of methemoglobinemia, a decrease in red cell glutathione, and a fall in hematocrit. In addition, there was a fall in the ratio of fluorescamine-reactive amino groups to lipid phosphorus in the C:I extracts. The latter finding is consistent with the covalent binding of carbonyl compounds to lipid amino groups, although other explanations cannot be precluded.

Separation of red cells according to density was performed by the centrifugation procedure of Murphy (28) on a sample obtained from this patient after 35 days of therapy. As shown in Table II, the bulk of the fluorescence was extractable from the denser reticulocyte-poor fraction, presumably representing older red cells. One interpretation of this observation is that the fluorescent cross-links accumulate during the life-span of the red cell, reflecting the continued production of MDA. Alternatively, the results may be due to a greater suscepti-

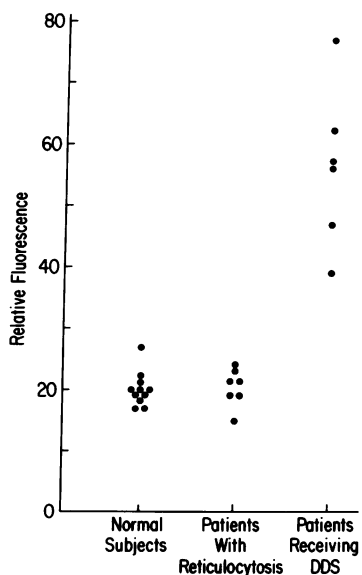


FIGURE 3 Fluorescence (excitation 360 nm; emission 440 nm) of C:I extracts of red cells obtained from normal subjects, patients with reticulocytoses, and patients treated with DDS.

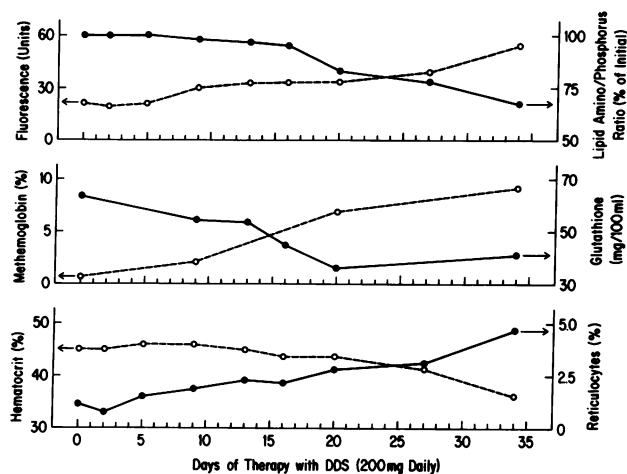


FIGURE 4 Laboratory findings in a patient with dermatitis herpetiformis treated with DDS.

bility of older red cells to DDS-induced lipid peroxidation.

The TBA test for free MDA in freshly obtained blood was negative in all subjects ( $< 2$  nmol MDA/ml). Serum vitamin E levels were in the normal range (560–1,420  $\mu\text{g}/100$  ml).

*Phenylhydrazine-treated rabbits.* C:I extracts of red cells obtained from four New Zealand white rabbits (2–3 kg) receiving phenylhydrazine, 1 mg/kg triweekly for at least 3 wk (courtesy of Dr. M. Freedman), revealed fluorescent emission spectra similar to that depicted in Fig. 1. Mean fluorescence at 440 nm was 131 U (range 62–210 U). In contrast, three control rabbits had 440 nm fluorescence of 25, 28, and 34 U in C:I extracts of their red cells.

## DISCUSSION

Daily therapy with DDS in the range of 50 mg or greater usually results in a mild Heinz body hemolytic

TABLE II  
Fluorescence in C:I Extracts of Red Cells Obtained from a Patient Receiving DDS after Separation by Density Centrifugation

Position in tube	Reticulocyte count	MCHC	Fluorescence
	%	%	U
Top	9.2	28.5	37
Bottom	2.8	33.2	68

The top of the centrifuge tube contains young red cells as indicated by the higher reticulocyte count and lower mean corpuscular hemoglobin concentration (MCHC).

process and elevated methemoglobin levels (29–32). As with other sulfone derivatives, individuals with red cell glucose-6-phosphate dehydrogenase deficiency are more susceptible to the hemolytic effects of this drug (33–34). The red cell toxicity of DDS is believed to be due to metabolites formed during its microsomal oxidation (35–36). Incubation of red cells with a DDS-hydroxylamine derivative has been shown by Glader and Conrad to result in the formation of intracellular hydrogen peroxide, as well as reproducing the hemolytic and methemoglobinemic effects observed in patients (37).

Recent studies by Rasbridge and Scott, which evaluated red cells obtained from individuals on long-term DDS therapy including the demonstration of a decrease in red cell GSH and stimulation of the hexose monophosphate shunt (38). These authors further suggested that cell membrane damage was the cause of DDS-induced hemolysis and, on the basis of *in vitro* incubation studies, hypothesized that lipid peroxidation had occurred (39). Their data, suggestive of lipid peroxidation, included a positive peroxide lysis test, which was partially prevented by the addition of vitamin E but not by sulfhydryl compounds, and a loss of acetylcholinesterase activity, a red cell membrane enzyme that has previously been shown to be susceptible to lipid peroxides. In addition, after incubation of whole blood from DDS-treated patients for 48 h at 37°C, the TBA test became positive in all eight subjects tested. However, it is of note that the TBA test was consistently negative before incubation. Furthermore, it is conceivable that evidence of lipid peroxidation observed after *in vitro* hemolysis may represent a posthemolytic artifact, inasmuch as ghosts appear to be far more sensitive to hydrogen peroxide-induced lipid peroxidation than are intact red cells and since hemoglobin solutions can catalyze the decomposition of lipid peroxides (40).

In the present study, the suggestion of Rasbridge and Scott, that DDS produces *in vivo* lipid peroxidation, has been supported by the demonstration of fluorescent spectra consistent with the cross-linking of lipid amino groups by carbonyl compounds derived from the breakdown of unsaturated fatty acids. This fluorescence was observed in extracts of freshly obtained red cells despite a negative TBA test. As the fluorescence assay presumably depends upon detection of dicarbonyl breakdown products of lipid peroxidation which have complexed with amino groups during the lifetime of the circulating red cell, it has an advantage, as compared to the TBA test, of being able to assess a cumulative *in vivo* event occurring over a relatively long period of time. Accordingly, it would appear that the fluorescence assay provides a useful method of detecting *in vivo* red cell lipid peroxidation.

The consequences to the circulating red cell of the covalent cross-linking of membrane contents are not

known. Current concepts of the dynamics of the red cell membrane have stressed its fluidity, particularly in relationship to the traversal of splenic sinusoids (41–42). It is conceivable that the cross-linking of membrane molecules would result in a decrease in membrane fluidity, thereby contributing to the splenic destruction of red cells during hemolysis provoked by oxidant drugs.

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