Erythropoietic Protoporphyria: Lipid Peroxidation and Red Cell Membrane Damage Associated with Photohemolysis

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ABSTRACT The mechanism by which long wavelength ultraviolet light hemolyzes red cells obtained from patients with erythropoietic protoporphyria (EPP) was investigated. Previous studies had suggested that irradiation of these red cells with wavelengths of light capable of eliciting dermatological manifestations led to oxygen-dependent colloid osmotic hemolysis through the formation of peroxides. In the present report, lipid peroxidation during in vitro irradiation of EPP red cells with long ultraviolet light was demonstrated by: (*a*) the formation of 2-thiobarbituric acid reactants; (*b*) the presence of conjugated diene bonds in red cell lipid; and (*c*) the selective loss of unsaturated fatty acids proportional to the number of carbon-carbon double bonds in each. Irradiation of EPP red cells was also shown to result in the formation of hydrogen peroxide.

Before photohemolysis there was a decline in cell membrane sulfhydryl groups and a loss in activity of the cell membrane enzyme acetylcholinesterase. These parameters provide further evidence suggesting that the cell membrane is a primary site of the photohemolytic effect of long ultraviolet light in EPP red cells.

Further evaluation of the radiation-induced inactivation of EPP red cell acetylcholinesterase was performed by radiating mixtures containing bovine erythrocyte acetylcholinesterase and protoporphyrin IX. These studies revealed that the rate of decline in enzyme activity is accelerated by the addition of linoleic acid, an unsaturated fatty acid, but not by palmitic acid, a saturated fatty acid.

Partial protection against both photohemolysis and acetylcholinesterase decline is provided by alpha-tocopherol. This lipid antioxidant loses its activity during the irradiation of EPP red cells suggesting that it is utilized in this process.

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INTRODUCTION

Erythropoietic protoporphyria (EPP)[1] is an inherited disorder of porphyrin metabolism in which the major clinical manifestation is cutaneous photosensitivity to wavelengths of light in the long ultraviolet range. Characteristic biochemical findings in this disorder, delineated by Magnus, Jarrett, Prankerd, and Rimington in 1961, include elevated protoporphyrin concentrations in red cells and feces (1). In 1964 Harber, Fleischer, and Baer demonstrated that long UV irradiation of red cells obtained from patients with EPP produced hemolysis (2). The light absorption spectrum of protoporphyrin is identical to the action spectrum eliciting both cutaneous photosensitivity and photohemolysis suggesting that the excitation of the protoporphyrin molecule by long UV light is responsible for cellular damage (1, 3).

The pathway by which the interaction of light and protoporphyrin might result in cell damage has been studied in this and other laboratories by investigation of EPP red cells subjected to radiation in vitro (3–8). It was shown that intracellular potassium loss and an increase in osmotic fragility preceded hemolysis, and that addition of nonpermeable osmotic solutes such as sucrose prevented hemolysis but not potassium loss (3).

[1] *Abbreviations used in this paper:* AChE, acetylcholinesterase; DFP, diisopropyl fluorophosphate; DTNB, dithiobisnitrobenzoic acid; EPP, erythropoietic protoporphyria; MDA, malonaldehyde; RBC, red blood cell; SH, sulfhydryl; TBA, 2-thiobarbituric acid; UFA, unsaturated fatty acids.

In 1967 Ludwig, Bilheimer, and Iverson presented evidence in abstract form that the photohemolysis of EPP red cells was associated with the formation of lipid peroxides and with a loss of intracellular glutathione (5). These authors hypothesized that red cell membrane damage caused by the light-induced excitation of protoporphyrin was due to the production of free radicals or hydrogen peroxide, which in turn resulted in the oxidation of unsaturated fatty acids (UFA) in the cell membrane. Studies in this laboratory, and by others, demonstrating that EPP photohemolysis will not occur in the absence of oxygen are consistent with Ludwig's hypothesis (7, 8).

The present investigation has utilized additional approaches to confirm the observation of lipid peroxidation during the photohemolysis of EPP red cells. We have also evaluated hydrogen peroxide formation, vitamin E protection, and the photolability of two constituents of the red cell membrane, SH groups and acetylcholinesterase, in EPP red cells irradiated with long UV light.

METHODS

Preparation and irradiation of red cell suspensions. Venous blood was obtained from five subjects with EPP, two of whom were related. Free erythrocyte protoporphyrin levels, measured by the method of Wranne (9), ranged from 700 to 1360 μg/100 ml (normal < 40 μg/100 ml). Hematocrits and reticulocyte counts of these patients were within normal limits. Disodium EDTA was used as an anticoagulant in order to chelate heavy metals which might possibly catalyze peroxidative or free radical events. Blood was centrifuged at 3,000 *g* for 10 min at 4°C, the plasma and buffy coat removed, and the red cells were washed three times in isotonic phosphate-buffered (0.01 M) saline, pH 7.4. Unless otherwise indicated, the erythrocytes were suspended in phosphate-buffered saline to a final packed cell volume of 1% before irradiation. A portion of this suspension was kept in the dark for use as a control sample.

Studies were also performed utilizing normal erythrocytes containing artificially elevated free protoporphyrin levels after incubation with protoporphyrin IX (pseudo-EPP red cells). These erythrocytes were obtained from normal volunteers and prepared as described above. The washed red cells were diluted to a 1% suspension in phosphate-buffered saline, pH 7.4, containing 500 μg/100 ml protoporphyrin IX (Calbiochem, Los Angeles, Calif.). After incubation for 30 min in the dark the erythrocyte suspension was centrifuged and the supernate discarded. The red cells were resuspended in buffer to the appropriate concentration for use on the same day. Care was taken to shield both EPP and pseudo-EPP red cells from light during the preparative procedures. Protoporphyrin IX was chemically homogeneous when assayed by the thin-layer chromatographic method of Ellfolk and Sievers (10).

Irradiation of red cell suspensions was performed with a bank of four 20-w black light tubes (General Electric Company, Schenectady, N. Y., No. F20T12-BL) with an emission of 400 nm (range 320–450 nm). The red cell suspensions were positioned approximately 7 cm from the radiation source in either slowly rotating 200-ml tonometry flasks or in beakers with constant magnetic stirring. The intensity of the radiation reaching the red cell suspension was approximately 1100 μw/cm². Temperature was maintained below 30°C. A monocellular layer of red cells is not achieved in this system and therefore a denser red cell suspension will have fewer photon hits per red cell at a given intensity of radiation.

Acetylcholinesterase and photohemolysis studies. Red cell acetylcholinesterase (AChE) was assayed by the colorimetric procedure of Ellman, Courtney, Andres, and Featherstone (11). Standard techniques were utilized for the measurement of hemoglobin, osmotic fragility, and hematocrit.

Vitamin E, in the form of *d*-alpha-tocopherol acetate was prepared on the day of use by mixing with 0.1 ml Tween 80 (polyoxyethylene sorbitan monooleate) per mg of alpha-tocopherol and then diluting with phosphate-buffered saline to a final concentration of 100 μg/ml. It was measured by the Emmerie-Engel reaction according to the procedure of Bieri, Teets, Belavady, and Andrews (12).

Bovine erythrocyte AChE (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.1 M phosphate buffer, pH 8.0, and frozen in portions until the day of use. It was then added in a final concentration of 4 μg/ml to a solution of protoporphyrin IX in the same buffer. When indicated, linoleic or palmitic acid (Mann Research Labs. Inc., New York) was added to this mixture after emulsification in Tween 80 (2 mg fatty acid per 0.1 ml Tween 80) in a final concentration of 0.1 mg/ml. In addition to linoleic acid, one sample contained alpha-tocopherol, 10 μg/ml, prepared in the same emulsion. The volume of the solutions was adjusted so that the concentration of protoporphyrin IX before irradiation was 2 μg/ml. Lipid-free control samples contained equivalent amounts of Tween 80. Linoleic acid had not peroxidized before use as determined by the 2-thiobarbituric acid test (13).

Lipid peroxidation assays. The peroxidation of red blood cell membrane lipids during irradiation with 400 nm light was evaluated by three techniques. The 2-thiobarbituric acid (TBA) test was performed by the method of Sinnhuber and Yu (13), as modified by Wills (14). In addition, the absorbance of the reaction mixture was read by scanning between wavelengths 500–560 nm to insure that a sharp peak was present at 532 nm. This is the absorption peak of malonaldehyde (MDA), a breakdown product of the peroxidation of polyunsaturated fatty acids which is considered to be responsible for the pink chromogen formed in the TBA test (15). The data were recorded as nanomoles MDA per milliliter erythrocytes. Malonaldehyde bis(diethylacetal) was used to obtain a standard curve.

The formation of conjugated diene bonds was evaluated by an adaption of the method of Recknagel and Ghoshal (16). Red blood cell lipid was extracted with isopropanol and chloroform according to the procedure of Rose and Oklander (17). Both reagents were redistilled and rendered carbonyl-free before use (18). In preliminary studies it was found that this method of lipid extraction was preferable to the chloroform and methanol technique (Procedure III) of Ways and Hanahan (19). The latter method resulted in the detection of conjugated diene bond formation in lipid extracted from fresh normal human erythrocytes in three of five preliminary studies. This presumed technical artifact may be due to the greater number of steps in the chloroform-methanol extraction procedure or to the more frequent contamination of the lipid sample with hemoglobin pigments. After extraction, the lipid sample was placed in a weighed round-bottom flask and the solvent evaporated

in a rotary evaporator (Buchler Instruments, Inc., Fort Lee, N. J.) under nitrogen. Chromatography grade carbonyl-free methanol was added to a final lipid concentration of 0.1 mg/ml and the UV absorbance spectrum recorded. In each study, lipid was extracted from parallel samples of irradiated and nonirradiated EPP red blood cells. In addition to obtaining the UV absorption spectrum of the individual lipid sample against a blank of methanol, a difference spectrum was provided by reading the irradiated sample with the nonirradiated sample as blank in a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The presence of a peak at 235 nm was taken as an indication of conjugated diene bond formation. All extraction procedures were performed under nitrogen to prevent artifactual lipid peroxidation.

Red blood cell fatty acids were measured by the method of Phillips, Dodge, and Rockmore in which intact erythrocytes are incubated in 2 N HCl for 18 hr at 110°C (20). The fatty acids are then extracted with pentane and methylated by the procedure of Morrison and Smith (21). Fatty acid analysis was performed by gas chromatography on a Varian aerograph model No. 1200 (Varian Associates, Walnut Creek, Calif.) and calculated by triangulation in comparison with standard fatty acid mixtures.

Hydrogen peroxide studies. Hydrogen peroxide production was measured indirectly by the method of Cohen and Hochstein in which the activity of endogenous erythrocyte catalase is utilized as a detection system (22). This assay depends upon the inhibition of a complex of catalase and hydrogen peroxide by aminotriazole (3-amino-1,2,4-triazole; Sigma Chemical Co., St. Louis, Mo.). As this compound will not inhibit catalase in the absence of hydrogen peroxide, the rate of decline in catalase activity is therefore proportional to the amount of hydrogen peroxide produced (23).

Another erythrocyte enzyme, glutathione peroxidase, will also decompose hydrogen peroxide thereby preventing its reaction with catalase (24). This difficulty can be partially obviated by preincubating washed erythrocytes at 37°C in the absence of an energy source. The resulting depletion of intracellular glucose removes substrate for glucose-6-phosphate dehydrogenase and therefore prevents the regeneration of NADPH required as a cofactor for the reduction of oxidized glutathione (GSSG) by the enzyme glutathione reductase. As glutathione peroxidase requires GSH for its function, in the relative absence of GSH a larger proportion of the hydrogen peroxide produced is free to react with catalase (24).

In the present studies, EPP erythrocytes were preincubated at 37°C in the dark for 3 hr before the beginning of the experiment. Aminotriazole was added to a final concentration of 50 mM. Catalase assays were performed by titration with permanganate according to the method of Tudhope (25).

Cell membrane sulfhydryl groups. Cell membrane SH groups were measured by the method of Szeinberg and Clejan which is based upon the reaction of dithiobisnitrobenzoic acid (DTNB) with SH groups resulting in the formation of a chromogen with peak absorption at 412 nm (26). This procedure measures only those cell membrane SH groups accessible to DTNB which was reported by Szeinberg and Clejan to amount to 13% of the total (26).

Cell membrane SH groups were determined in irradiated 10% suspensions of EPP erythrocytes. At 4 and 6 hr of irradiation, duplicate 20-ml portions were removed and centrifuged at 25,000 *g* for 20 min. After discarding the supernate, ghosts were prepared by the method of Dodge, Mitchell, and Hanahan (27). Samples of the cell membrane preparation were removed for determination of residual hemoglobin by the benzidine method (28), and for measurement of total protein by the procedure of Lowry, Rosebrough, Farr, and Randall (29). Cell membrane SH levels were related to ghost protein levels corrected for residual hemoglobin content.

RESULTS

Acetylcholinesterase. In Fig. 1 the effect of continuous 400 nm irradiation on the osmotic fragility and AChE of a 1% EPP red cell suspension is shown in solid lines. There is a progressive loss in AChE activity which initially precedes a significant increase in osmotic fragility and occurs before overt colloid osmotic hemolysis.

The relationship of long UV light to the loss of AChE was evaluated by placing a portion of the EPP red cell suspension in the dark after 60 min of irradiation while the remaining sample was continuously irradiated. As shown in Fig. 1 irradiation for 60 min results in sufficient damage to the EPP red cell so that osmotic hemolysis continues despite termination of the radiation exposure, although at a somewhat slower rate than in those red cells which are continuously radiated. In contradistinction, no further loss of AChE activity occurs after removal of the EPP red cells from the radiation source despite eventual hemolysis.

Similar results were obtained when pseudo-EPP erythrocytes were exposed to light. In control studies, irradiation of normal erythrocytes had no effect on AChE levels, nor did incubation of EPP or pseudo-EPP red blood cells in the dark affect enzyme activity. AChE levels in five subjects with EPP ranged from 9.6 to 11.2 μmoles/min per ml red cells which is within normal limits for this procedure (11).

Studies were performed evaluating the possible radiation-induced formation of an inhibitor of AChE. The AChE activity of equal mixtures of irradiated and nonirradiated EPP red cell suspensions incubated in the dark for 30 min was equivalent to the values predicted if no inhibitor were present (Table I). Incubation of nonirradiated EPP red cells in cell-free supernate prepared from irradiated EPP red cell suspensions, and dialysis of the irradiated EPP red cell suspensions for 18 hr at 4°C and 25°C with 1,000 volumes of isotonic phosphate-buffered (0.01 M) saline, pH 7.4, also failed to reveal evidence of an inhibitor (Table I). These experiments only exclude a stable, extracellular, dialyzable inhibitor.

The relationship of the activity of AChE to photohemolysis was studied in irradiated EPP red cells previously incubated with the AChE inhibitor diisopropyl fluorophosphate (DFP; 0.01 mM). No discern-

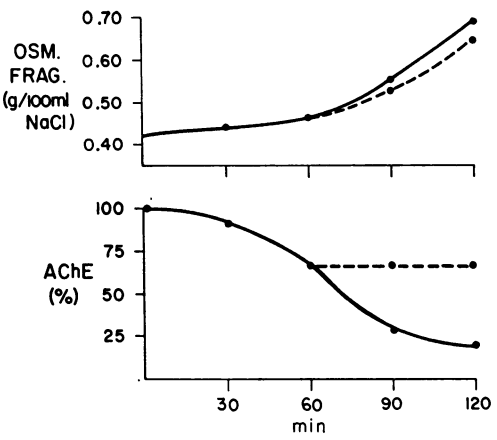

FIGURE 1 Osmotic fragility (Osm. Frag.) and acetylcholinesterase (AChE) of irradiated 1% EPP red cell suspensions. The dashed lines indicate the data in a portion of the red cell suspension placed in the dark after 60 min of radiation. Osmotic fragility is expressed as g/100 ml sodium chloride at which 50% hemolysis occurs and AChE as the percentage of the initial value. Results represent the average of three experiments.

ible difference in the rate of photohemolysis was detectable in EPP red cells with no AChE activity as compared to a similar EPP red cell suspension not incubated with DFP.

Thiobarbituric acid reactants. Thiobarbituric acid reactants (TBA) were measured in irradiated 1% EPP red blood cell suspensions. The data in Table II represent the averages of four experiments. After 30 min of irradiation there is only a slight elevation of TBA reactants as compared to the preradiation value. A statistically significant increase ($P < 0.05$) in levels of TBA reactants is noted after 60 min of irradiation at which time 4% of the erythrocytes had hemolyzed and AChE activity had declined to 44% of initial activity. Continued irradiation resulted in further increases in TBA reactants. Similar results were obtained with irradiated pseudo-EPP red blood cells. No significant increase in TBA reactants was observed during dark incubation of EPP erythrocytes nor after irradiation of red cells having normal protoporphyrin concentrations.

Conjugated diene bond formation. The UV absorption spectra of lipid extracted from irradiated and nonirradiated EPP erythrocytes are shown in Fig. 2. Conjugated diene bond formation in the irradiated samples is indicated by the absorption peak at 235 nm. In this study 5% suspensions of EPP erythrocytes were irradiated for 4 hr or incubated in the dark. The irradiated red blood cell suspension had 2% hemolysis and contained 64% of its initial AChE activity. Conjugated diene bonds were also demonstrable in pseudo-EPP erythrocytes under similar conditions. However, diene

TABLE I

*Acetylcholinesterase Activity of Irradiated and Nonirradiated 5% EPP Red Cell Suspensions after Mixing or Dialysis**

Sample	AChE
	% of initial activity
Nonirradiated control (A)	100
Irradiated for 120 min (B)	32
Incubated equal mixtures of A and B‡	68
Incubated A cells with B supernate‡	98
Incubated B cells with A supernate‡	30
Dialysis of A at 4°C§	96
Dialysis of A at 25°C‡	93
Dialysis of B at 4°C‡	30
Dialysis of B at 25°C‡	29

* Data given are average of two experiments.

‡ Incubations performed at 25°C for 60 min.

§ Red cell suspensions dialyzed with 1,000 volumes of phosphate-buffered saline, pH 7.4, for 18 hr.

TABLE II

TBA Reactants in 1% EPP Red Cell Suspensions Irradiated with Long UV Light

Time	TBA Reactants	Hemolysis	AChE
min	*nmoles MDA/ml RBC ±SE*	*%*	*% of initial activity*
0	4.1 ±0.5	0	100
30	4.9 ±1.3	0	78
60	8.3 ±2.9	4	44
120	32.6 ±8.8	28	24

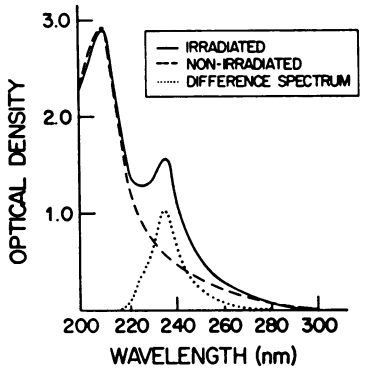

FIGURE 2 Conjugated diene bond formation in irradiated EPP red cell lipid. The solid line indicates the UV absorption spectrum of lipid (0.1 mg/ml methanol) extracted from a 5% suspension of EPP erythrocytes irradiated for 4 hr with 400 nm light. Lipid extracted from a nonirradiated 5% EPP red cell suspension is shown by the dashed line. The dotted line represents the difference spectrum between these two samples. The presence of conjugated diene bonds is indicated by the peak at 235 nm.

conjugation could not be consistently demonstrated in lipid extracted from 5% suspensions of pseudo-EPP or EPP erythrocytes irradiated for lesser periods of time and containing more than 75% of their initial AChE activity. Conjugated diene bond formation was not observed in lipid extracted from normal human erythrocytes irradiated for 4 hr with 400 nm light.

Red cell fatty acid composition. Additional evaluation of the peroxidative breakdown of UFA was obtained by quantitating red cell fatty acids in irradiated and nonirradiated samples by gas chromatography. Red blood cell fatty acids were measured in duplicate 5% suspensions of EPP erythrocytes irradiated for 6 hr. In both samples, 10% of the red blood cells had hemolyzed at the cessation of irradiation. Duplicate control suspensions of EPP erythrocytes were incubated in the dark for 6 hr. The five major red blood cell fatty acids were measured in triplicate in each sample and the data were pooled for statistical analysis of the effect of irradiation. A significant decrease in UFA was noted in the irradiated suspensions (Table III). This decline is approximately proportional to the number of carbon-carbon double bonds in each UFA (oleate 18:1, linoleate 18:2, and arachidonate 20:4; declined 7, 12, and 21%, respectively). The saturated fatty acid palmitate was essentially unchanged after irradiation, however, there was a 3% decline in stearate (18:0), which is statistically significant ($P < 0.05$). A slight loss of stearate has also been observed in other systems in which erythrocyte lipid peroxidation has been documented (30–32). It is unlikely that the loss in UFA is solely due to a posthemolysis artifact as destruction of all the arachidonic acid in the 10% of the erythrocytes that hemolyzed could not account for the 21% loss of this polyunsaturated fatty acid.

In a similar experiment, 400 nm irradiation of pseudo-EPP erythrocytes (RBC-free protoporphyrin 540 μg/100 ml) also resulted in a specific loss of UFA approximately proportional to the number of double bonds in each (Table IV). The apparent loss in stearic acid was again observed, although without statistical significance ($0.20 > P > 0.10$). At the cessation of the 6 hr radiation period in this study, 28% hemolysis had occurred. An additional control of normal red cells not incubated with protoporphyrin IX was evaluated. Irradiation of a 5% suspension of these red cells with 400 nm light for 6 hr did not result in any significant change in red cell fatty acids.

TABLE III
Major Fatty Acid Composition of Irradiated and Nonirradiated EPP Red Cells

Fatty acid	Nonirradiated*	Irradiated*	Loss
			%
16:0	31.0 ±0.8	30.4 ±0.4	1.7
18:0	24.5 ±0.5	23.5 ±0.7	3.9‡
18:1	22.6 ±0.5	21.0 ±0.6	7.1§
18:2	13.9 ±0.9	12.3 ±0.4	11.6§
20:4	23.7 ±0.8	18.6 ±1.0	21.3§

* Mg × 10^{-12} per RBC ±SE.
‡ $P < 0.05$.
§ $P < 0.01$.

TABLE IV
Major Fatty Acid Composition of Irradiated and Nonirradiated Pseudo-EPP Red Cells

Fatty acid	Nonirradiated*	Irradiated*	Loss
			%
16:0	31.8 ±0.5	31.8 ±0.6	0
18:0	24.0 ±1.1	23.0 ±0.8	4.0
18:1	22.3 ±0.4	20.5 ±0.7	8.3‡
18:2	14.2 ±0.6	11.9 ±0.6	16.2‡
20:4	22.7 ±0.4	16.8 ±0.5	26.1‡

* Mg × 10^{-12} per RBC ±SE.
‡ $P < 0.01$.

Hydrogen peroxide formation. Irradiation of a 1% suspension of EPP red cells containing 50 μM aminotriazole produced a relatively rapid decline in catalase activity as compared to the nonirradiated control (Table V). This finding is consistent with the formation of hydrogen peroxide during 400 nm radiation of EPP red cells. The rate of loss of catalase activity was greater than the rate of hemolysis. In the absence of aminotriazole, catalase activity declined by 12% in a suspension of EPP red cells irradiated for 180 min.

A loss in catalase activity was also observed in irradiated pseudo-EPP red cell suspensions containing aminotriazole. However, no significant decline in catalase was noted in normal red cells irradiated in the presence of aminotriazole. Red cell catalase levels in four subjects with EPP were 0.38–0.45 × 10^{-8} moles per ml RBC which is within the normal range reported by Tudhope (25).

Vitamin E studies. The protective effect of vitamin E was evaluated by measuring photohemolysis and AChE activity in irradiated 1% suspensions of EPP red cells containing 10 μg/ml alpha-tocopherol emulsified in Tween 80. Control 1% EPP red cell suspensions contained equivalent amounts of Tween 80 but no alpha-tocopherol. These studies indicate that vitamin E provides partial protection against both radiation-induced photohemolysis and AChE inactivation (Fig. 3). The lower portion of Fig. 3 represents the levels of alpha-tocopherol detectable during the irradiation of EPP red

TABLE V

*Catalase Activity and Percentage Hemolysis in Irradiated and Nonirradiated EPP Red Cell Suspensions Containing 50 μM Aminotriazole**

Minutes	Catalase		Hemolysis	
	Irradiated	Nonirradiated	Irradiated	Nonirradiated
	% of initial activity		%	
30	98	100	1	0
60	92	99	2	1
90	81	98	8	1
120	62	97	24	1
180	19	94	62	2

* Data given are average of three experiments.

cell suspensions to which this antioxidant had initially been added. There is a marked decline in the activity of this exogenous vitamin E resulting in a level of 1.0 μg/ml detectable after 4 hr of exposure to long UV light. Two additional control suspensions containing 10 μg/ml alpha-tocopherol were utilized in this latter study. The vitamin E levels of a 1% suspension of normal red cells irradiated for 4 hr was 8.2 μg/ml; and of a 1% suspension of EPP red cells incubated in the dark for 4 hr was 9.2 μg/ml.

Cell membrane sulfhydryl groups. Long UV irradiation of a 10% suspension of EPP red cells resulted in a decline in DTNB-reactive cell membrane SH groups from a preradiation level of 37.5×10^{15}/mg cell membrane protein to 22.4×10^{15}/mg protein at 4 hr and 14.1×10^{15}/mg protein at 6 hr (Table VI). This represents a per cent loss of cell membrane SH groups at 4 hr of 40% and at 6 hr of 62% which is almost as rapid as the corresponding decline in AChE activity. This loss of DTNB-reactive cell membrane SH groups preceded significant hemolysis as only 13% of the EPP red cells had hemolyzed during the 6 hr of irradiation.

No correction was applied for the SH groups of residual hemoglobin in the cell membrane preparations as it was not known to what extent they were oxidized during irradiation. Assuming no oxidation of hemoglobin SH groups, the data would indicate a lesser loss in cell membrane SH groups after irradiation than actually occurred as there was generally a slightly greater hemoglobin contamination in ghosts prepared from the irradiated samples. The opposite assumption of complete oxidation of hemoglobin SH groups would lead to an overestimation of the decline of cell membrane SH in the present study. However, recalculation of the data based on either of these assumptions results in at most a 5% overestimation or 8% underestimation of the relative amount of cell membrane SH groups present after 6 hr of irradiation.

TABLE VI

DTNB-Reactive SH Groups in Irradiated EPP Red Cell Membranes

Minutes	SH groups*	Hemolysis	AChE
0	37.5 ±2.2	0	100
240	22.4 ±3.6	4	52
300	14.1 ±4.8	13	32

* Expressed as 10^{15} SH groups per milligram ghost protein.

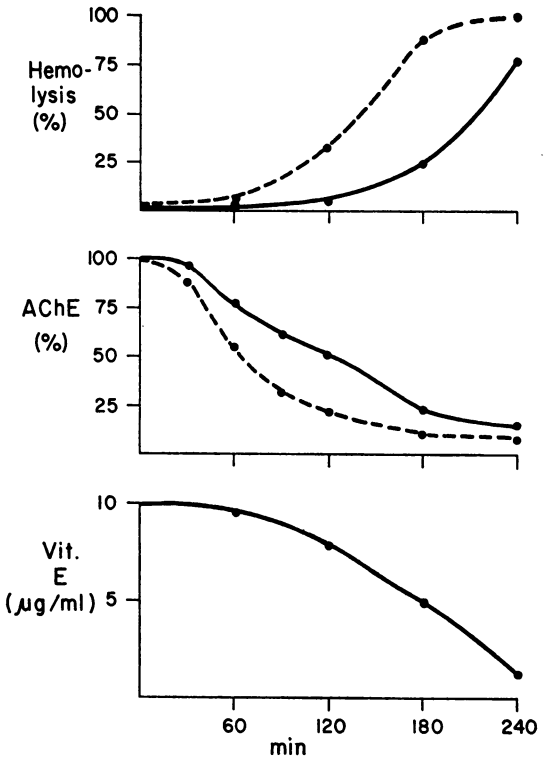

FIGURE 3 The protective effects and utilization of vitamin E in 1% suspensions of EPP red cells irradiated with 400 nm light. Vitamin E (alpha-tocopherol acetate) was emulsified in Tween 80 and added to a final concentration of 10 μg/ml. Control EPP red cell suspensions contained equivalent amounts of Tween 80 but no vitamin E. In the top and middle figures, the percentage hemolysis and the per cent of initial AChE activity in the EPP red cell suspensions containing vitamin E is indicated by the solid lines and in the control EPP red cell suspensions by the dashed lines. The lower curve demonstrates the measurable levels of vitamin E in the EPP red cell suspensions to which vitamin E had been added. The data represent the average of three experiments.

Neither incubation of EPP red cells in the dark nor irradiation of normal red cells for 6 hr had any significant effect on cell membrane SH groups.

Bovine erythrocyte acetylcholinesterase study. The relationship of the decline in AChE to lipid peroxida-

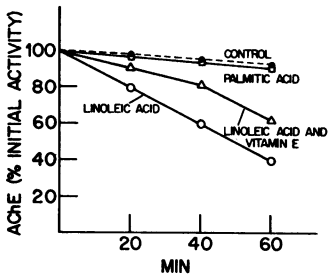

FIGURE 4 The effect of fatty acid saturation and vitamin E on the activity of bovine erythrocyte AChE (4 μg/ml) irradiated with 400 nm light in a solution containing protoporphyrin IX (2 μg/ml). Palmitic acid (16:0) and linoleic acid (18:2) were emulsified in Tween 80 and added to a final concentration of 0.1 mg/ml. One sample contained vitamin E (alpha-tocopherol acetate, 10 μg/ml) in addition to linoleic acid. An equivalent amount of Tween 80 was present in the control sample. The data represent the average of two experiments.

tion was evaluated in a system utilizing bovine erythrocyte AChE. In this study a mixture of protoporphyrin IX (final concentration 2 μg/ml) and bovine erythrocyte AChE (final concentration 4 μg/ml) received 400 nm radiation for 60 min and the AChE activity determined. Little loss of enzyme activity was demonstrated in this control sample (Fig. 4). However, the addition to this mixture of linoleic acid (0.1 mg/ml) produced a marked decline in AChE levels during irradiation which could be partially prevented by the further addition of alpha-tocopherol (10 μg/ml). In contradistinction, addition of the saturated fatty acid palmitate to the AChE-protoporphyrin mixture did not produce a significant acceleration of loss of enzyme activity. Identical nonirradiated experimental mixtures did not demonstrate any change in AChE activity during a parallel 60 min incubation in the dark. An exception was the AChE-protoporphyrin mixture containing linoleic acid in which a 6% decline in enzyme activity was observed. Irradiation of bovine erythrocyte AChE in buffer was without effect.

DISCUSSION

Two different wavelengths of light have been demonstrated to result in the photohemolysis of human red cells. Hemolysis due to UV light in the 300 nm range is ascribed to energy absorption by protein chromophores causing direct damage to the red cell (33). Production of hemolysis by long UV light in the range of 400 nm was originally described by Cook and Blum with relatively high total dosage required for normal red cells (34). Based on the absorption characteristics of this phenomenon and the kinetics of the reaction, these authors hypothesized that hemolysis caused by 400 nm light was produced indirectly by the photoexcitation of a nonprotein chromophore. They further suggested that the photosensitizer might be free erythrocyte protoporphyrin.

The increased susceptibility to photohemolysis of EPP red cells, which contain free erythrocyte protoporphyrin levels 10–100 times those of normal, is in accord with this hypothesis. The only other known difference between normal red cells and EPP is the slightly higher levels of coproporphyrin in the latter. However, previous studies of red cells with markedly increased coproporphyrin levels, such as found in lead poisoning, have shown no evidence of in vitro photohemolysis or clinical photosensitivity (4, 35). It is therefore unlikely that coproporphyrin plays a role in this phenomenon.

The mechanism of the photosensitization process by which visible light is transformed into chemical energy has been extensively studied in recent years. The primary event is the absorption of light by the photosensitizer resulting in the formation of an excited molecular state. There are several possible pathways by which photoexcitation of one molecule may cause oxidation of an adjacent molecule that would otherwise be unaffected by light. These include the formation of free radicals, peroxides, singlet oxygen, and charge transfer complexes (36, 37). Based on studies of photosensitized lysosomal damage, Slater and Riley suggested that the biochemical mode of action of a photosensitizer was the initiation of free radical reactions of the peroxy type with resultant damage to cellular membranes (38). Studies of the photochemistry of porphyrins include the observation of photoinduced porphyrin free radicals by Mauzerall and Feher (39), the detection of electron transfer from irradiated protoporphyrin IX (8), and the demonstration of the photodynamic action of porphyrins on amino acids and proteins (40).

These biophysical characteristics of the photoactivation of protoporphyrin and the previous demonstration of the oxygen-dependence of EPP photohemolysis suggest that peroxide formation has a major role in red cell damage (8). The relative lipid solubility of protoporphyrin further suggests that excitation of this molecule by 400 nm radiation can lead to transfer of energy within the lipid milieu of the cell membrane. Among the molecular constituents of the cell membrane theoretically most susceptible to such a process are the carbon-carbon double bonds of UFA.

The oxidative breakdown of UFA results not only in the disruption of the fatty acid molecule, but also in the production of a number of highly reactive intermediates, including free radicals and carbonyl compounds, presumably capable of causing cell damage (41, 42). Lipid peroxidation has been implicated as a mechanism of biological damage in a number of diverse entities including

vitamin E deficiency (43), carbon tetrachloride and ethanol hepatotoxicity (16, 44), X-irradiation (45), ozone and nitrogen dioxide toxicity (46, 47), kinky hair disease (48), and aging (49). The association of the oxidative breakdown of cell membrane UFA with hemolysis has been noted in studies of normal and vitamin E–deficient erythrocytes exposed to oxidizing agents (30, 50–52). Mengel and his coworkers, in a series of investigations evaluating the hemolytic effects of hyperbaric oxygenation, demonstrated that lipid peroxidation preceded hemolysis both in vitro and in vivo (53, 54). These authors also noted the concomitant loss of red cell AChE activity and demonstrated the susceptibility of this cell membrane enzyme to preformed lipid peroxides (55, 56). Erythrocyte lipid peroxidation and inhibition of AChE has also been demonstrated after exposure to the oxidant air pollutant ozone (57, 58). Red cell lipid peroxidation and inactivation of cell membrane SH groups have also been shown to be concomitants of the in vitro hemolytic effects of ionizing radiation (45, 59).

The present studies confirm the previous report by Ludwig et al. suggesting that lipid peroxidation occurs during the photohemolysis of EPP red cells (5). These authors assayed lipid peroxidation by the TBA technique which depends primarily on the formation of MDA during the breakdown of polyunsaturated fatty acids. This procedure is not specific for MDA as TBA will also react with a number of other compounds (60, 61). Furthermore, Hochstein and Ernster found that only about 5% of peroxidized rat liver microsomal lipid was split into MDA (62).

In the present study, two additional techniques were utilized to demonstrate that 400 nm radiation of EPP red cells results in lipid peroxidation. The presence of conjugated diene bonds in lipid extracted from irradiated EPP red cells was detected by UV spectrophotometry. This procedure has as its major drawback the lack of quantitative relationship between the spectrophotometric measurements and the extent of lipid peroxidation. The remaining technique, the demonstration of a selective loss of UFA upon measurement of red cell membrane fatty acids, would appear to be the most valid indicator of lipid peroxidation in this system. Membrane damage unassociated with the oxidation of UFA would presumably result in an indiscriminate loss of all fatty acids.

In the present study, the demonstration that the loss in individual unsaturated fatty acids is approximately proportional to the number of double bonds at risk (oleic, 7%; linoleic, 12%; arachidonic, 21%) is consistent with a process in which excitation of protoporphyrin by 400 nm light results in an oxygen-dependent electron transfer to the double bonds of cell membrane UFA producing lipid peroxidation.

The association of lowered AChE activity with lipid peroxidation is in accord with previous studies (55–58). Further evaluation of AChE in radiated EPP red cells demonstrated that cessation of irradiation after 1 hr resulted in no further loss in AChE activity despite continued colloid osmotic hemolysis. These findings suggest that inhibition of AChE is directly dependent on the photoexcitation of protoporphyrin. The possible relationship of lowered AChE activity to lipid peroxidation in this system is suggested by the accelerated loss of enzyme activity when bovine erythrocyte AChE and protoporphyrin IX is irradiated in the presence of linoleic acid. The failure of DFP, an inhibitor of AChE, to effect the rate of photohemolysis is in accord with previous studies suggesting that AChE enzyme function is not of critical importance to the integrity of the red cell (63).

Ludwig et al. demonstrated that preincubation of EPP red cells with tocopherol protected against lipid peroxide formation, hemolysis, and GSH decline during irradiation (5). In addition, they noted low plasma tocopherol levels in subjects with EPP and presented evidence that vitamin E deficiency appeared to enhance EPP photohemolysis but was not solely responsible for this phenomenon. These authors further suggested that there was an increased utilization of this antioxidant in subjects with EPP. The present studies demonstrate that alpha-tocopherol affords partial protection against both photohemolysis and AChE inactivation. The decline in activity of this antioxidant during irradiation of EPP red cells further suggests that vitamin E is utilized in vitro.

In addition to lipid peroxidation and AChE inhibition, the present studies indicate that 400 nm irradiation of EPP red cells results in a loss in DTNB-titrable cell membrane SH groups and in the production of hydrogen peroxide. The oxidation of SH groups is a documented effect of free radicals and lipid peroxides (64). Jacob and Jandl have shown that the inhibition of red cell SH groups leads to colloid osmotic hemolysis which depends primarily upon membrane SH groups rather than intracellular GSH (65). The loss of cell membrane SH groups in irradiated EPP red cells may thus be at least partially responsible for photohemolysis.

Hydrogen peroxide formation during long UV irradiation of EPP red cells was suggested by previous studies in which horseradish peroxidase was utilized as an indicator (8). However, horseradish peroxidase will also react with organic peroxides while catalase, used in the present investigation, is specific for hydro-

gen peroxide. The hemolytic effects of hydrogen peroxide have been shown to be associated with lipid peroxidation (52). The formation of hydrogen peroxide, possibly resulting from free radicals derived from the excitation of protoporphyrin, may thus be responsible for lipid peroxidation and hemolysis in irradiated EPP red cells. However, as the breakdown of UFA may itself produce hydrogen peroxide (66), the presence of hydrogen peroxide in this system might be secondary to lipid peroxidation.

It is therefore difficult to assess the relative importance of lipid peroxidation, hydrogen peroxide formation, and cell membrane SH loss in the photohemolytic process. The partial protection against photohemolysis provided by vitamin E suggests that the breakdown of UFA does contribute to the photohemolytic process. Similarly, partial protection afforded by SH protective agents reported both in the red cell system (7) and in an in vivo animal model (67) is consistent with a role for SH oxidation in the hemolysis of irradiated EPP red cells. It is likely that all of these processes contribute to cell damage during the radiation of EPP red cells with long UV light.

In addition to lipid and hydrogen peroxides, it is theoretically possible that a protoporphyrin peroxide is also formed during the oxygen-dependent photohemolysis of EPP red cells. The difficulty in assaying this presumably highly unstable intermediate has precluded evaluating this possibility in a biological system.

The production of free radicals by 300 nm UV light has been demonstrated in human skin (68) and is believed to be the basis of the biological effects of this part of the light spectrum. The mechanism by which 400 nm light results in the dermatological manifestations of EPP is still subject to experimental evaluation. However, it should be noted that long UV light is capable of penetrating into the epidermis, dermis, and vasculature of the skin. Furthermore, there are a number of possible pathways by which protoporphyrin formed in erythropoietic or hepatic tissues may reach these layers (69). It is plausible that the process by which EPP red cells undergo photohemolysis in vitro may also account for the mechanism by which 400 nm light produces the clinical cutaneous changes in this disorder.

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