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STUDIES OF PHOTOPROTECTION AGAINST PORPHYRIN PHOTOSENSITIZATION USING DITHIOTHREITOL AND GLYCEROL*

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ABSTRACT

Although protection against ionizing radiation by compounds containing sulfhydryl (SH) groups, such as cysteine, has been reported, these agents have been unsuccessful to date in protecting mammals against non-ionizing radiation (> 200 nm). This study describes successful photoprotection by dithiothreitol (DTT) and glycerol against porphyrin photosensitization having an action spectrum of 400 nm. Test models used were red blood cells (RBC) obtained from patients with erythropoietic protoporphyria (EPP) and mice photosensitized by hematoporphyrin (HP).

A mortality rate approaching a lethal dose in 50% of the animals in 1 day (LD_{50}/24 hrs) was established in 100 white mice that had received an intraperitoneal injection of 100 mg HP/kg body wt., and were then irradiated with 5×10^6 ergs/cm^2 from a fluorescent light source emitting 320–450 nm radiation. Another 100 mice were treated in an identical manner except that they received, in addition to HP, 80 mg DTT/kg body weight in a 5.5% glycerol solution. This group showed 75% reduction in mortality ($p < 0.03$). No lethal effects were observed in animals treated with DTT and glycerol or HP in the above concentrations without 400 nm irradiation.

RBC obtained from patients with EPP and exposed to 10^7 ergs/mm^2 of 400 nm radiation showed 100% hemolysis after 180 min. These cells, when irradiated under identical conditions except for the addition of DTT, manifested only 19% hemolysis during this period of time. Measurements of SH groups of RBC from patients with EPP showed a progressive decrease during photohemolysis. Comparison of the rate of photohemolysis of normal and glucose-6-phosphate dehydrogenase (G6PD) deficient RBC irradiated in the presence of protoporphyrin IX revealed that G6PD deficient RBC, which have an impaired ability to produce reduced glutathione (GSH), were more susceptible to porphyrin-induced photohemolysis.

These studies demonstrate that DTT and glycerol offer photoprotection in an *in vivo* mammalian system against porphyrin photosensitization. It is suggested that the mechanism of the photoprotective action against 320–450 nm radiation has many features similar to that of radioprotection by thiols and glycerol against ionizing radiation.

Numerous studies have dealt with protection by sulfhydryl-containing (SH) compounds against the deleterious effects of x-rays. These have been comprehensively reviewed by Bacq (1) and others (2). Following the classical radioprotective studies with cysteine by Patt in 1949 (3), the ability of thiols to protect against the effects of this type of ionizing radiation has been documented in many systems. Far fewer studies have dealt with protection by SH-containing compounds against ultraviolet or visible light photosensitization. The use of these compounds as photoprotective agents against long ultraviolet light (400 nm) has been controversial, with photoprotection being reported in an *in vitro* system (4, 5) and negative results in *in vivo* mammalian (6, 7) studies.

In the present investigation, *in vivo* photoprotection against porphyrin photosensitization was assessed in mice and in an *in vitro* human red blood cell (RBC) photohemolysis model. The thiol compound chosen for photoprotection, dithiothreitol (DTT), 2,3-dihydroxy-1,4-dithiolbutane (Fig. 1), has a low redox potential (8) and contains 2 moles of sulfhydryl groups per mole of DTT. As noted in Figure 2, (8), DTT absorbs no photons in the 400 nm range which is the absorption spectrum peak of protoporphyrin.

Recently, Sanner and Pihl reported data concerning an "indirect effect" of glycerol in protecting *E. coli* against ionizing radiation (9). Additional reports of radioprotection by glycerol have been reviewed by Bacq (10). Further studies (11) have indicated a cumulative and/or synergistic effect when 2 radioprotective agents were used concurrently. Accordingly, DTT was evalu-

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CLELAND'S REAGENT

DITHIOTHREITOL (DTT)

2,3-dihydroxy-1,4-dithiobutane

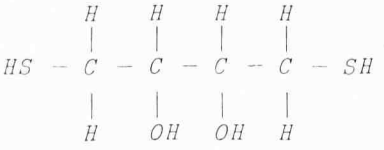

FIG. 1. Legend on print

ULTRAVIOLET SPECTRUM OF OXIDIZED DTT AND DTT IN WATER

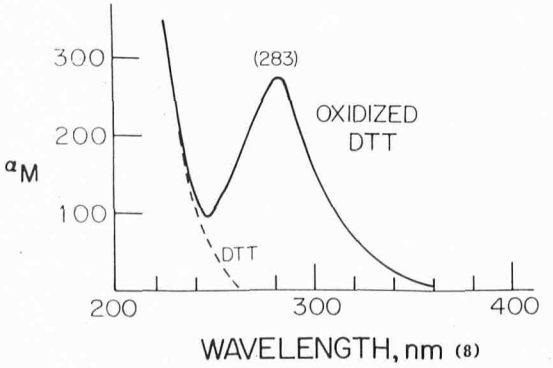

FIG. 2. Legend on print

ated for ultraviolet photoprotection both in the presence and absence of glycerol.

MATERIALS AND METHODS

Red Blood Cell Studies

Protection against *in vitro* photohemolysis of cells from patients with erythropoietic protoporphyria was studied using techniques previously described (12, 13) with minor modifications.

Source and preparation of erythrocytes. Erythrocytes were obtained from 3 patients with erythropoietic protoporphyria who had free erythrocyte protoporphyrin levels ranging from 900–1000 μg/100 ml of packed RBC as determined by the method of Wranne (14). The normal value is less than 35 μg/100 ml. The RBS of an unaffected volunteer with an average protoporphyrin level of 27 μg/100 ml of packed RBC served as controls. Both types of RBC were washed three times and suspended in a 1:400 dilution in isotonic phosphate buffered (0.01 M) saline, pH 7:4. This buffer was used in all *in vitro* studies.

Photoprotective assays. 1) DTT Photoprotection Assay in RBC: A monocellular RBC suspension was approximated by placing 1.0 ml of RBC in a 3 ml quartz cuvette 2.5 cm in diameter with an optical pathway of 1 cm. The suspension was prepared by adding equal amounts of EPP RBC (1:400 dilution) and DTT (26.6 mM) made up in buffer. The buffer without DTT was added to an aliquot of the EPP RBC suspension to serve as a control. Both cuvettes were then exposed to 400 nm radiation from a solar simulator (15) using the apparatus previously described (12). Photohemolysis was assayed by means of direct cell count in a hemocytometer before, during and after exposure. Suspensions identical to those irradiated were kept in the dark as additional controls. Direct cell counts in a hemocytometer were made prior to monolayer formation which occurred when RBC were allowed to settle for 30 min. 2) Glycerol Photoprotection Assay in RBC: 1.0 ml of an EPP RBC suspension (1:800 dilution) with 10% glycerol by volume was irradiated with controls and was treated identically as described in the DTT photoprotection assay. 3) DTT and Glycerol Photoprotection Assay in RBC: 0.5 ml glycerol (20%) was added to 0.5 ml EPP RBC (1:400 dilution) to yield 1.0 ml EPP RBC (1:800 dilution) solution suspended in DTT (10.7 mM) with 10% glycerol by volume. Control samples and irradiation techniques were as described above.

Determination of thiol alterations during photohemolysis. Red cells glutathione (GSH) and photohemolysis were assayed in 1% EPP RBC suspensions irradiated with the fluorescent tube light source (13). RBC suspensions were placed in slowly rotating 250 ml flasks positioned approximately 15 cm from the light source. Control suspensions were kept in the dark. For determination of GSH and percentage hemolysis, 20 ml aliquots of the RBC suspensions were centrifuged at 5000 × g for five minutes at 3° C and the supernatants removed for hemoglobin determination. The remaining RBC were immediately diluted to a final volume of 2 ml in buffer for measurement of GSH by the colorimetric method of Beutler, *et al.* (16). Percentage hemolysis was calculated by relating the concentration of supernatant hemoglobin to that of the uncentrifuged 1% suspension. In view of the low hematocrit, no correction was made for the volume of distribution of supernatant hemoglobin. Glutathione concentrations were corrected for the extent of hemolysis using the following formula: GSH (corrected) = GSH (observed)/1 − % hemolysis. This was necessary as preliminary studies had shown that no GSH was present in the supernatant of the irradiated EPP RBC suspensions and that GSH (10 mg/100 ml) added to an EPP red cell suspension during photohemolysis was not detectable within ten minutes. These observations led to the assumption that all GSH measured in the irradiated RBC suspensions was within nonhemolyzed red cells.

Glucose-6-phosphate dehydrogenase deficient erythrocytes. Glucose-6-phosphate dehydrogenase (G6PD) deficient RBC were obtained from two Negro males. Enzyme activity was 0.5 and 0.6 international units (17). The subjects were otherwise hematologically normal with no clinical evidence of hemolysis. Pseudo-EPP RBC were prepared simultaneously from these RBC and from RBC obtained from a hematologically normal volunteer using our previously described technique (18). Both samples were irradiated as described above.

Animal Studies

Determination of hematoporphyrin phototoxicity in mice. Swiss Webster strain female albino mice weighing 20–28 gms were used throughout the study. Hematoporphyrin, obtained from Calbiochem and prepared according to the method of Lipson and Baldes (19), was injected intraperitoneally into the animals 30 minutes prior to irradiation. The source of 400 nm radiation consisted of a bank of 4 blacklight fluorescent tubes having

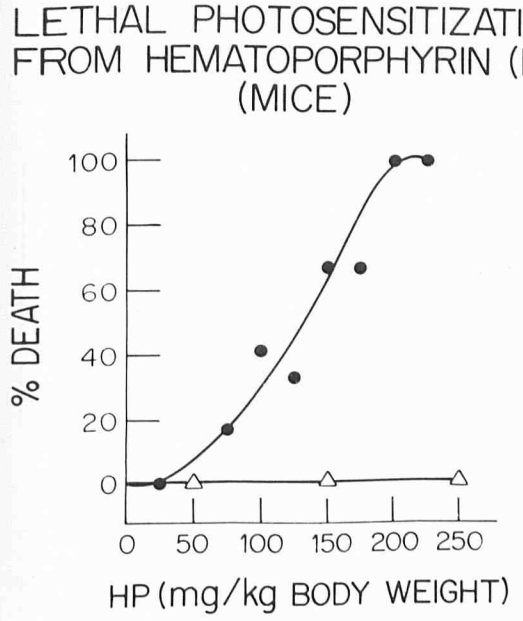

FIG. 3. Legend on print

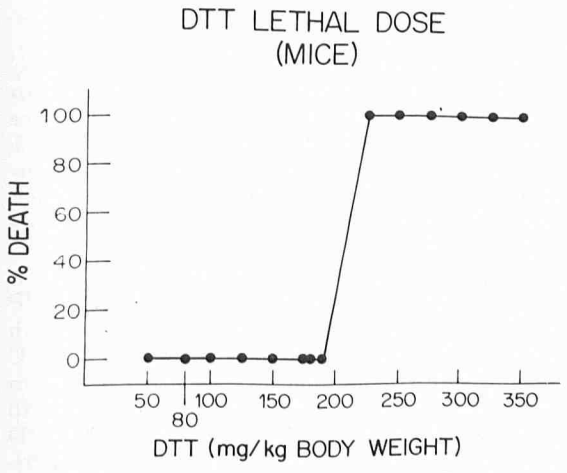

FIG. 4. Control studies of DTT toxicity. Each represents at least six animals.

an emission spectrum ranging from 320–450 nm. Radiation factors were: target skin distance 12 cm; radiation time 120 min.; total energy 5.01×10^6 ergs/cm^2. Pilot studies established the dose of hematoporphyrin that would have a lethal effect on 50% of the irradiated group in 24 hours (LD_{50}/1 day). This was accomplished by injecting groups of at least 6 mice with hematoporphyrin concentrations ranging from 25 to 250 mg/kg body weight. A dose of 100 mg of hematoporphyrin/kg body weight was established as the LD_{50}/1 day and used in all further studies (Fig. 3).

Toxicity studies of DTT and glycerol. Dithiothreitol (DTT) was assessed for toxicity in mice. Care was taken to keep the experimental animals protected against light following intraperitoneal injection of DTT. This compound was found to be lethal to mice in concentrations greater than 190 mg/kg of body weight (Fig. 4). Glycerol was assayed for toxicity in similar studies and found to be lethal at a 15% concentration in volumes less than 1 ml (Fig. 5). Accordingly, DTT at a dose of 80 mg/kg was injected intraperitoneally in isotonic phosphate buffered saline to which glycerol had been added to a final concentration of 5.5%. Studies with DTT in this saline glycerol vehicle showed no lethal effect (Fig. 6).

Photoprotection studies using DTT and glycerol. Two hundred mice were irradiated *in vivo*. Group 1 consisted of 100 animals that received hematoporphyrin and 30 min. later light exposure while Group 2, also consisting of 100 animals, received hematoporphyrin followed immediately by 80 mg/kg DTT in a 5.5% glycerol-phosphate-buffered saline solution. Thirty min. later, Group 2 animals received light exposure. The mice were irradiated in groups of 12 animals on separate days, 6 from Group 1 and 6 from Group 2. Each animal received a total dose of 5×10^6 ergs/cm^2. No anesthesia was required during the irradiation as the animals were restrained in 0.5 inch wire mesh holders. Viability of the animals was assessed at intervals of 1 and 2 days.

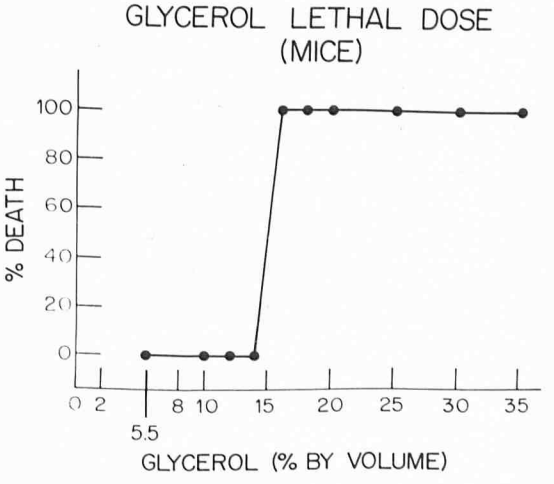

FIG. 5. Control studies of DTT and glycerol toxicity. Each represents at least six animals.

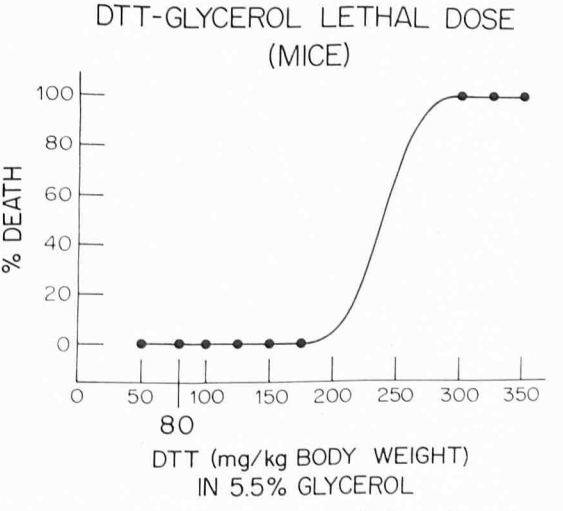

FIG. 6. Control studies of DTT and glycerol toxicity. Each represents at least six animals.

Photoprotection against HP by DTT or glycerol alone. The photoprotective effects of 5.5% glycerol alone were assessed in 60 mice, using an intraperitoneal injection of 5.5% glycerol and a dose of radiation of 5×10^6 ergs/cm^2. Similar studies evaluating the photoprotective effects of 80 mg/kg DTT, in the absence of glycerol, were done in 48 mice.

RESULTS

Red Blood Cell Studies

Figure 7 is based on direct RBC counts in a hemocytometer before, during and after exposure to 400 nm irradiation from a "Solar Simulator". Cells from patients with EPP showed 100% photohemolysis within a period of 180 minutes. Addition of glycerol to the RBC suspension had only negligible effect on photohemolysis. However, when DTT was added to the RBC suspension, only 19% photohemolysis was noted during the first 3 hours. The addition of both glycerol and DTT in the above concentrations induced greater photoprotection, thus suggesting a synergistic effect which was apparent at 5 hrs. Observation over an 8 to 10-hour period also indicated greater photoprotection with both of these agents than when either agent was used alone. Control studies of RBC obtained from normal individuals or patients with EPP whose RBC were treated with these agents but were not irradiated showed only negligible hemolysis during a 10-hour period.

Table I demonstrates the loss of intracellular sulfhydryl groups, measured as GSH, from RBC of EPP patients that were irradiated with 400 nm radiation. Inactivation of GSH occurred more rapidly than hemolysis, since there was a 92% decline in GSH and 33% hemolysis at 180 minutes of irradiation. There was no change in EPP RBC GSH levels during parallel incubation in the dark.

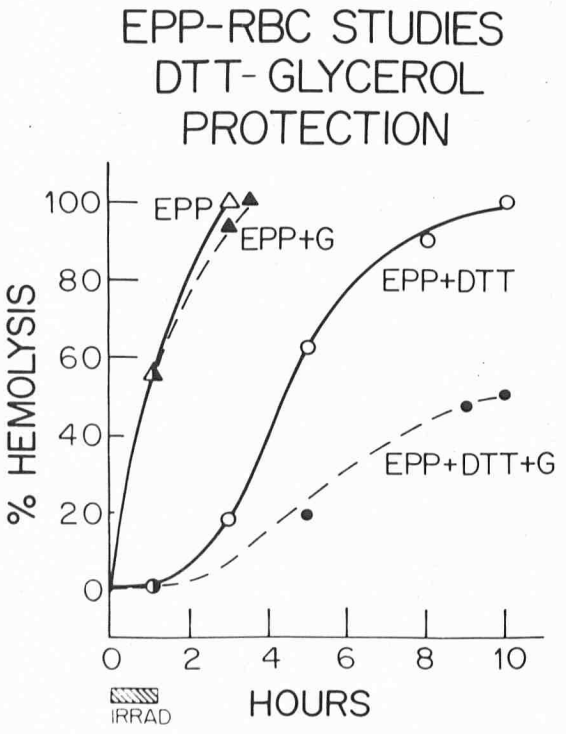

FIG. 7. Photoprotection by DTT and glycerol against photohemolysis by DTT and glycerol. Data obtained by direct RBC counts.

TABLE I

GSH levels and photohemolysis in EPP red cells receiving 400 nm radiation

Time (minutes)	GSH (% loss)	Hemolysis (%)
60	12	2
120	44	12
180	92	33
240	99	88

Average of 2 experiments.

TABLE II

Effect of 400 nm radiation on GSH levels and rate of hemolysis in pseudo-EPP normal erythrocytes and pseudo-EPP G6PD deficient erythrocytes

Minutes of irradiation	GSH (% loss)		Hemolysis (%)	
	Normal RBC	G6PD deficient RBC	Normal RBC	G6PD deficient RBC
60	16	52	3	17
120	52	92	15	45
180	99	100	40	100

The rate of inactivation of GSH and the hemolysis of G6PD deficient and normal pseudo-EPP RBC irradiated with 400 nm light is shown in Table II. The G6PD deficient pseudo-EPP RBC demonstrate a more rapid decline in GSH and an accelerated rate of hemolysis as compared to pseudo-EPP RBC containing normal G6PD levels. Control studies showed no significant diminution in GSH levels nor hemolysis when pseudo-EPP G6PD deficient or pseudo-EPP normal RBCs were incubated in the dark for 180 minutes; nor when G6PD deficient or normal RBC not previously incubated with protoporphyrin IX were irradiated with 400 nm light.

Mice

Figure 3 indicates the results of the assay of hematoporphyrin phototoxicity. Additional studies of the toxicity of DTT (Fig. 4), glycerol (Fig. 5), and DTT and glycerol (Fig. 6) demonstrated that an intraperitoneal dose of 80 mg/DTT/kg body weight in a 5.5% glycerol phosphate buffered saline solution was nontoxic to the experimental animals. In the photoprotection studies the mice that had received 80 mg HP/kg body weight and irradiation had a survival rate of 59%, whereas those that had received the same

TABLE III
PROTECTION AGAINST PORPHYRIN PHOTOSENSITIZATION
LD_{50}/ 24 hr.

	DEATH	SURVIVAL
GROUP 1 (CONTROL)	41	59
GROUP 2 (DTT-GLYC.)	10	90

$p = < 0.03$

dose of HP, irradiation as well as DTT and glycerol, had a 90% survival rate ($p < 0.03$) (Table III). Studies in 60 mice receiving 5.5% glycerol alone showed no photoprotective effect. This finding parallels similar findings in RBC photohemolysis studies. When DTT was studied alone in 24 mice, fewer fatalities were noted in the group receiving this SH agent as compared to 24 control animals (8/24 vs. 11/24). However, this finding is not statistically significant.

DISCUSSION

Erythropoietic protoporphyria, identified by Magnus *et al.* in 1961 as an inborn error of protoporphyrin metabolism, is usually transmitted in an autosomal dominant genetic pattern (20). It is characterized by marked photosensitivity occurring in the 400 nm range, which closely corresponds to the absorption spectrum peak of the protoporphyrin molecule. Recent reports have suggested that in addition to synthesis of protoporphyrin by erythroid elements, the liver also participates in manufacture of this photosensitizer (21). The cutaneous lesions appear in early childhood and may present a varied morphology, in most cases consisting of painful edematous plaques on the light exposed areas. Frequently there is a history of a marked "burning" sensation before clinical appearance of the lesions (22).

Previous studies by our group (13, 18, 23) and others (4, 5, 24, 25) have demonstrated that RBC from patients with EPP offer a useful experimental model for studying the mechanisms of cell damage from 400 nm irradiation. These *in vitro* studies have shown that the photohemolysis system exemplifies cell membrane damage mediated by a colloid osmotic hemolysis mechanism (5, 23). Photohemolysis with 400 nm irradiation was also shown to be oxygen dependent (4, 26, 27) and associated with peroxide formation (25, 27). Further studies by our group (28) and others (25) have shown that following free radical formation in an oxygen environment, cell membrane lipid peroxides as well as hydrogen peroxide may be detected by numerous biochemical assays. Whether or not the peroxide formed is protoporphyrin peroxide, hydrogen peroxide, or a cell membrane lipid peroxide, these agents can each potentially cause lytic lesions in the membrane. The size of the membrane lesion has been estimated in previous studies to be less than 10 Å (23). *In vitro* studies have demonstrated the formation of free radicals following excitation of hematoporphyrin (29), uroporphyrin and coproporphyrin (30).

There are theoretically three broad pathways through which chemicals may exert photoprotective properties in this system: a) photoprotection by absorbing or blocking the 400 nm photons which excite porphyrins; b) photoprotection by secondary mechanisms that divert the energy following excitation of porphyrins before it is transferred to the cell and c) repair of cell damage caused by photoactivated porphyrins and their products. Evidence has also recently been obtained suggesting that protective agents may affect the intrinsic photosensitization properties of a cell (31).

Protection by thiol derivatives. As recently reviewed, numerous thiol derivatives have been shown to be effective radioprotective agents (2, 32). By extension of these classical radioprotection studies to porphyrins, it is proposed that following photo-excitation of protoporphyrin, dithiothreitol acts by protecting cell structures against free radicals formed from water and other organic sources (33). In addition to protecting by trapping free radicals, photoprotection may also occur where a hydrogen atom is split from a vital molecule through a process of hydrogen donation. It is also reasonable to extend Bacq and Alexander's original radioprotection observations (1, 33) to include the concept that long ultraviolet wavelength excitation of porphyrins results in the formation of excited singlet oxygen and free radicals, which interact with H_2O and unsaturated fatty acids in the membrane to form peroxides. This is schematically illustrated in the following equations:

$$\text{I} \quad PP + H_2O \xrightarrow{400\ nm} PP^{\cdot} + H^{\cdot} + OH^{\cdot}$$

$$\text{II} \quad PP^{\cdot} + O_2 \longrightarrow PP + O_2^{*}$$

$$\text{III} \quad O_2^{*} + 2H_2O \longrightarrow 2H_2O_2$$

$$\text{IV} \quad O_2^{*} + H^{\cdot} \longrightarrow HO_2^{\cdot}$$

$$\text{V} \quad 2RSH + 2OH^{\cdot} \longrightarrow R{-}S{-}S{-}R + 2H_2O$$

$$\text{VI} \quad 2RSH + HO_2^{\cdot} \longrightarrow R{-}S{-}S{-}R + H_2O$$

Equation I indicates that protoporphyrin following 400 nm radiation is converted into a highly unstable and short-lived free radical which,

in the absence of O_2 (in an N_2 environment) causes no membrane lesion (27). However, as noted in Equations II, III and IV, in the presence of molecular O_2, the energy from the porphyrin radical will form an excited oxygen singlet state with resultant free radical $HO_2^{\cdot}$ and hydrogen peroxide as well as $H^{\cdot}$ and $OH^{\cdot}$ radicals. It is $HO_2^{\cdot}$ that is long-lived and potentially the most damaging (33). Equations V and VI indicate possible mechanisms through which thiols may protect by their preferential reactivity with radicals before they encounter a vital membrane target. As noted, the donation of hydrogen by thiols can convert the peroxy radical into inert H_2O, whilst the thiol becomes a disulfide. Thus the thiol function is a free radical "trapping agent" or a "radical scavenger". Although this theory of thiol radioprotection against x-rays has been stressed by Alexander (33) and Doherty (34) and does seem applicable to porphyrins excited by long ultraviolet, alternative hypotheses must be considered in a complex mammalian organism such as the mouse. Alternative mechanisms of thiol protection not directly related to the scavenging of free radicals have been summarized by Bacq (35) and may be applicable to protection against porphyrin photosensitization. They include: protein binding by thiols alters the cell per se to make it more radioresistant; disulfide products formed following irradiation may aid in repair; thiols bound to cellular protein may result in release of enzymes and other agents which contribute to photorepair and/or photoprotection. In addition, we have also irradiated DTT and glycerol in sera to insure that there were no significant shifts in its absorption spectrum to account for photoprotection in the 400 nm range.

Protection by glycerol. Glycerol has been known to protect against x-ray damage to bacteria (36) and yeast (37). The mechanism to date is not fully understood. Pihl and Sanner (38) suggested that a radical scavenger mechanism as well as hydrogen transfer are the key features of glycerol protection. It is also felt that glycerol (which is necessary in relatively high concentrations for protective effects as compared to thiols) may act by occupying vital sites on macromolecules, thus displacing H_2O (36). This obviously would decrease free radical formation at the surface of vital cell membrane chemical units. Thus the cell could be considered to have been made more resistant to radiation. In addition, in the present studies, a non-specific membrane effect of glycerol cannot be excluded.

The photoprotective effect of DTT against photohemolysis in EPP RBC was readily demonstrable with a 13.3 mM solution of this thiol. Recently, Suurmond and coworkers have also reported protection by thiols using dimercaptopropanol (BAL) and cysteine (4, 5). However, due to the differences in radiation factors, it is not possible to compare the relative effectiveness of these different thiols. At a concentration of 5.5% we were unable to demonstrate a protective effect of glycerol alone against photohemolysis. When the concentration was increased further, it was difficult to assay hemolysis. However, a synergistic effect was noted when glycerol and DTT were used together. Similar synergisms in radioprotective studies have been reported by others (39, 40). In particular, Hotz has reported that glycerol adds its radioprotective action to that of cysteine or cysteamine in T-1 bacteriophage (41). Whether these agents are acting by a similar or different protective mechanism remains conjectural.

Studies have previously demonstrated that the sulfhydryl groups of the RBC membrane are inactivated during irradiation of EPP RBC with 400 nm light and that loss of these sulfhydryl groups precedes hemolysis (28). In the studies presented here, the inactivation of intracellular sulfhydryl groups was demonstrated (Tables I and II). In addition, the importance of the normal red cell sulfhydryl protective mechanism in the defense against the products of activated protoporphyrin was indicated by a greater susceptibility of G6PD deficient pseudo-EPP RBC to undergo photohemolysis (Fig. 2). The relative absence of G6PD renders the RBC less able to produce NADPH, which is required as a co-factor to reduce oxidized glutathione (GSSG) back to reduced glutathione (GSH). This disorder is manifested clinically by an increased susceptibility to the hemolytic effects of various drugs which appear to have in common the ability to produce free radicals or peroxides. Accelerated photohemolysis and reduced GSH in G6PD deficient pseudo-EPP RBC is therefore compatible with a process in which the activation of protoporphyrin by 400 nm light results in the formation of free radicals and/or peroxides. It should be noted that ionizing radiation to red cells has been reported to result in potassium loss, decreased GSH, inactivation of cell membrane SH groups, lipid peroxidation, and eventual hemolysis; all of which has been demonstrated in EPP RBC radiated with 400 nm light (42, 43, 44, 45). Furthermore, SH-containing compounds are effective in protecting RBC against ionizing radiation (45, 46).

The studies in mice, which were designed to utilize the synergistic effect noted in the RBC studies, demonstrated that protection could also be obtained in an *in vivo* mammalian system. Here again, glycerol alone showed negligible protection. It must be stressed (Figs. 4 and 5) that both DTT as well as glycerol are toxic in mice, causing lethal effects at high concentration. It is not intended in this paper to extrapolate this data to man but rather to illustrate a principle that could potentially unite selected aspects of x-ray and ultraviolet protection. It has also been possible to demonstrate photoprotection in the visible light range effected by a thiol in a dye-photosensitized yeast system (47). Thus there is reason to believe that the fundamental features of

thiol photoprotection may have relevance in all forms of electromagnetic radiation. If it is established that free radical formation and peroxidation of cell membrane structures (25, 28) are the major routes initiating photosensitivity damage in vital cellular organelles, an extended search for non-toxic free radical scavengers as well as hydrogen transfer agents should be made, and suitable compounds should be evaluated in other photodermatoses (e.g., drug photosensitivity reactions) as well as in the various types of porphyria.

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