SELF-SENSITIZED PHOTO-OXIDATION OF PROTOPORPHYRIN IX AND RELATED PORPHYRINS IN ERYTHROCYTE GHOSTS AND MICROEMULSIONS: A NOVEL PHOTO-OXIDATION PATHWAY INVOLVING SINGLET OXYGEN†

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(Received January 5, 1984)

Summary

Previous studies have shown that protoporphyrin IX can sensitize its own photo-oxidation by paths involving primarily singlet oxygen and to a lesser extent superoxide (via excited state electron transfer). These reactions involve reaction of the vinyl groups of the protoporphyrin to yield porphyrins with modified side-chains. Quantum efficiencies are generally low and the product distribution is somewhat solvent dependent. In recent work we have examined the photo-oxidation of protoporphyrin IX, mesoporphyrin IX and hematoporphyrin IX in erythrocyte "ghosts", a natural membrane system containing saturated lipids, unsaturated lipids and a full complement of membrane proteins. We observe that all three porphyrins are rapidly photo-oxidized in the ghosts and that the products produced from protoporphyrin IX do not include the "usual" singlet oxygen products. We have been able to model the kinetic behavior observed in the natural membrane systems by using an oil–water microemulsion as a solvent medium and adding various amino acids that are easily oxidized. In particular we have investigated the behavior of methionine and a number of related thioether derivatives. The results of this study suggest that the porphyrins sensitize singlet oxygen efficiently but that the singlet oxygen is rapidly scavenged by substrates such as methionine and other amino acids. The oxygenated amino acids can subsequently act as agents to oxidize the porphyrins efficiently by attacking the porphyrin ring directly. Thus, although singlet oxygen is clearly indicated to be involved in these reactions, the actual agent in the

†Paper presented at the COSMO 84 Conference on Singlet Molecular Oxygen, Clearwater Beach, FL, U.S.A., January 4 - 7, 1984; paper 39 of the series Photochemical Reactions in Organized Assemblies.

0047-2670/84/$3.00

photo-oxidation of the porphyrins appears to be an intermediate occurring subsequent to its consumption.

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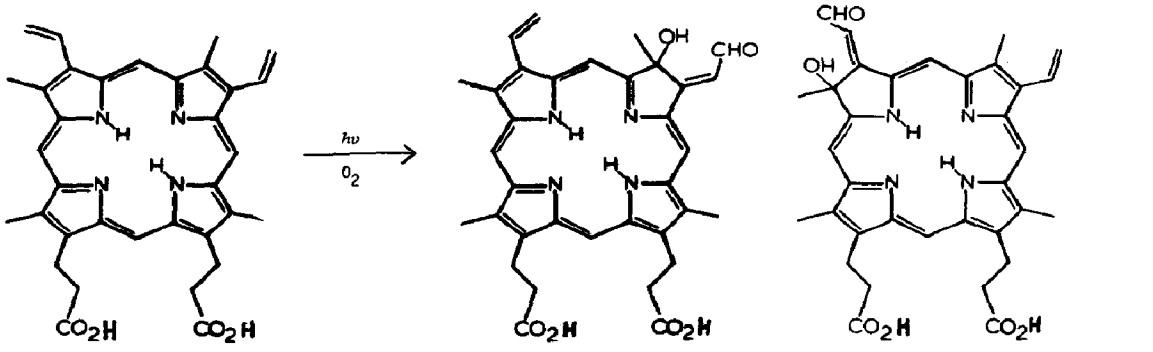
1. Introduction

Porphyrins and metalloporphyrins and related pyrrole pigments are well known to sensitize their own photo-oxidation in a variety of diverse reactions ranging from simple electron transfer through side-chain oxidation to oxidative cleavage of the tetrapyrrole macrocycle [1 - 8]. A variety of these reactions have been shown to involve primarily or exclusively singlet oxygen [4, 5, 7, 9, 10]; indeed a number of investigations have established that porphyrins and metalloporphyrins, via their triplet states, are efficient sensitizers of singlet oxygen [11 - 13]. The biologically important protoporphyrin IX free base is among those porphyrins particularly sensitive to self-sensitized oxidations; typical quantum yields for degradation of protoporphyrin IX (1) range from 0.033 in chloroform to 0.006 in benzene [14]. Through several investigations it has been established that the major products of protoporphyrin IX oxidation in these solvents are 2 - 6 [3, 9, 10]:



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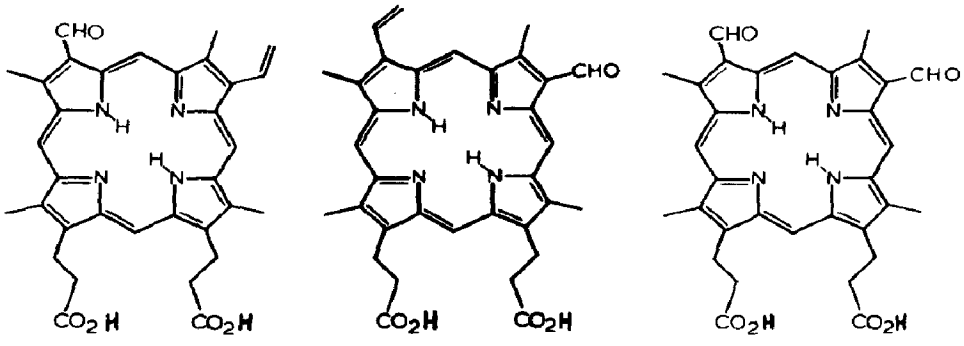

4 5 6 (1)

Investigations in detergent micelles and other microheterogeneous media have shown that these products are also formed in these media but in ratios which are quite medium sensitive [10]. Although the changes in product distribution observed for the photo-oxidation of 1 can be partially attributed to the occurrence of different paths for photo-oxidation, most notably an electron transfer quenching of the triplet or singlet states of 1 to produce superoxide radical anions [9, 15], it has been well established that each of the products 2 - 6 and indeed the major portion of the photoreaction in most "non-participating" solvents or "solvent environments" arise from direct attack of singlet oxygen on ground state porphyrin [10].

We have recently extended studies of the self-sensitized oxidation of 1 to different environments including erythrocyte "ghosts", a natural membrane system which consists of saturated and unsaturated lipids as well as membrane proteins [16]. These nearly colorless membrane fragments, produced by osmotic shock which releases hemoglobin and other cellular components contained within the cell membrane (we have used so-called "white non-resealed" ghosts) [17 - 19], readily incorporate a number of porphyrins and other dyes [16]. We have found that 1 can be incorporated as a monomeric species into ghosts and that irradiation of 1 leads to its rapid bleaching but to relatively little of the "normal" singlet oxygen photoproducts 2 - 6 [16]. Since the environment provided by the erythrocyte ghosts may be very similar to that occurring when free base 1 or related octaalkyl porphyrins such as hematoporphyrin IX or "hematoporphyrin derivative" (HPD) are incorporated into living cells, it might be anticipated that photoreactions occurring in the ghosts with 1 or hematoporphyrin IX might parallel "photodynamic action" phenomena occurring in the porphyrins [20, 21] or in cancer phototherapy [22 - 24].

In the present paper we report an investigation of the oxygen-induced photobleaching of several natural and synthetic porphyrins and metalloporphyrins in erythrocyte ghosts. We also report parallel studies of the photobleaching of the same porphyrins in an oil-in-water microemulsion which provides a somewhat similar solvent environment to the lipid portion of a natural membrane. This medium has been used to probe systematically the type of reaction occurring in the ghosts; by adding individually several ghost components we have been able to duplicate the pattern of photoreactivity occurring in the ghosts and to suggest a mechanism for the photobleaching in which singlet oxygen participates as an intermediate but in which the key attack resulting in oxidative degradation of the porphyrin involves intermediates formed subsequent to excited singlet oxygen.

2. Experimental section

2.1. Materials

1 was obtained from Calbiochem–Behring and used without further purification. Protoporphyrin IX dimethyl ester was synthesized as described

previously [9]. HPD was purchased from Porphyrin Products and used as received. Mesoporphyrin IX dimethyl ester (Sigma) was converted to the diacid as previously described [25]. Hematoporphyrin IX dimethyl ester was prepared using a literature preparation [26] while tetraphenylporphyrin and tetraphenylporphyrin zinc(II) were synthesized from pyrrole and benzaldehyde in proprionic acid [27 - 29]. The surfactants dodecyltrimethylammonium bromide (DTAB) and sodium dodecylsulfate (SDS) were obtained from Sigma and Biorad respectively. DTAB was recrystallized twice from acetone before use while SDS (electrophoresis grade) was recrystallized twice from absolute ethanol. The amino acids L(−)methionine and L(−)tryptophan were purchased from Aldrich and recrystallized once from ethanol. L(+)cysteine, L-histidine, L(+)arginine, D,L-norvaline and ethyl sulfide were obtained from Aldrich and used without further purification. Methionine sulfoxide was synthesized after the procedure of Sysak *et al.* [30]. *n*-butanol was Fisher (certified ACS, A-399). Water was triply distilled as described previously [31].

2.2. Erythrocyte ghosts

Ghosts were prepared as described by Parker and Hoffmann [17]. They were stored in a freezer and used within 2 - 3 weeks in each case. For incorporation of porphyrins 1 ml of ghost solution was thawed. The porphyrin was added dropwise as a tetrahydrofuran solution (60 - 100 μl) under stirring. The ghost solution was then stirred for 30 min in an open container to permit evaporation of the tetrahydrofuran. 4 ml of an iso-osmotic solution (0.15 M NaCl–0.01 M tris; pH 5 in water) was added and the mixture stirred for 1 h. The porphyrin concentration was $(1.5 - 2.0) \times 10^{-5}$ M.

After irradiation the ghost solutions were treated with a few drops of 0.1 M HCl to acidify the proprionic acid groups. The products were then extracted with a tetrahydrofuran:(diethyl ether) mixture (40:60) and then treated with an ether solution of diazomethane to afford the corresponding methyl esters. This enabled analysis of the starting porphyrin and products by high pressure liquid chromatography (HPLC).

Although the porphyrin methyl esters were generally not found to be incorporated into the ghosts cleanly as monomers, treatment of solutions prepared as described above, then cooled to 0 °C in an ice bath, with an ether solution of diazomethane, afforded ghost solutions which contained esterified porphyrin which remained monomeric. These solutions showed nearly identical irradiation behavior with those containing free proprionic acid groups. After irradiation the starting and product ester derivatives were extracted with a (40:60) tetrahydrofuran:ether mixture and subjected directly to HPLC analysis. Results obtained on the pre-esterified ghost-incorporated porphyrins were always, within experimental error, the same as those obtained by esterification following irradiation.

2.3. Microemulsion studies

The oil-in-water microemulsions were prepared from 1 g of surfactant (DTAB or SDS) (10.2 wt.%), 6 ml of water (61.1 wt.%), 2.4 ml *n*-butanol

(19.9 wt.%) and 1 ml benzene (or a 1 ml benzene solution containing the porphyrin) (8.9 wt.%). The porphyrin (dimethyl ester) concentration used in the microemulsion was (1.5 - 1.8) $\times 10^{-5}$ M. Amino acids or other "additives" were dissolved in the microemulsion to obtain the desired concentrations.

The microemulsion solutions were worked up for HPLC analysis by adding 1.5 g NaCl to 9 ml of microemulsion to cause phase separation. The organic layer (containing the porphyrin and other colored material) was collected and combined with two portions of benzene which were used to wash the aqueous phase. The combined organic solutions were evaporated on a rotary evaporator to dryness ($T < 35$ °C) and the residue dissolved in water was extracted three times with ethyl acetate and the combined organic extracts were evaporated for HPLC analysis.

2.4. Irradiation and analysis

Irradiations were carried out in a merry-go-round apparatus using a Hanovia 450 W medium pressure mercury lamp. Corning 0-52 filters were used to eliminate light of $\lambda < 366$ nm. HPLC analysis of photo-oxidation products was carried out using a Perkin–Elmer series 1 high pressure liquid chromatograph with a Varian UV–visible detector. A Whatman Partisil PXS 5/25 column was used with a solvent system of chloroform:hexane (70:30). The products were monitored with the detector set at 420 nm and the detector was calibrated using solutions of the photo-oxidation products synthesized separately as standards. UV and visible spectra were recorded on a Perkin–Elmer 576ST spectrophotometer. Ghost spectra were obtained in the turbid sample compartment with a non-porphyrin-containing ghost solution as a reference.

3. Results

3.1. Irradiation of oxygenated ghost solutions of porphyrins

Irradiation of 1 in air-saturated ghost solutions leads to bleaching of the porphyrin visible and near-UV bands. The rate of bleaching is close to that for 1 in homogeneous solution or microheterogeneous media such as DTAB micelles or dipalmitoylphosphatidylcholine (DPPC) vesicles (Fig. 1 and Table 1). Unlike the results observed in the other media, there is no build-up of absorption at 670 nm concurrent with the disappearance of 1 (Fig. 2); this behavior suggests that the green hydroxyaldehydes 2 and 3 are not being formed in the photobleaching.† HPLC analysis of ghost solutions containing 1 after irradiation also indicates very little formation of products 4 - 6 and reveals very little visible-light-absorbing species as products of the irradiation.

†The possibility that 2 and 3 are formed in the ghosts but subsequently rapidly degraded appears to be unlikely since other porphyrins, particularly mesoporphyrin IX, not having vinyl groups degrade at rates similar to that of 1.

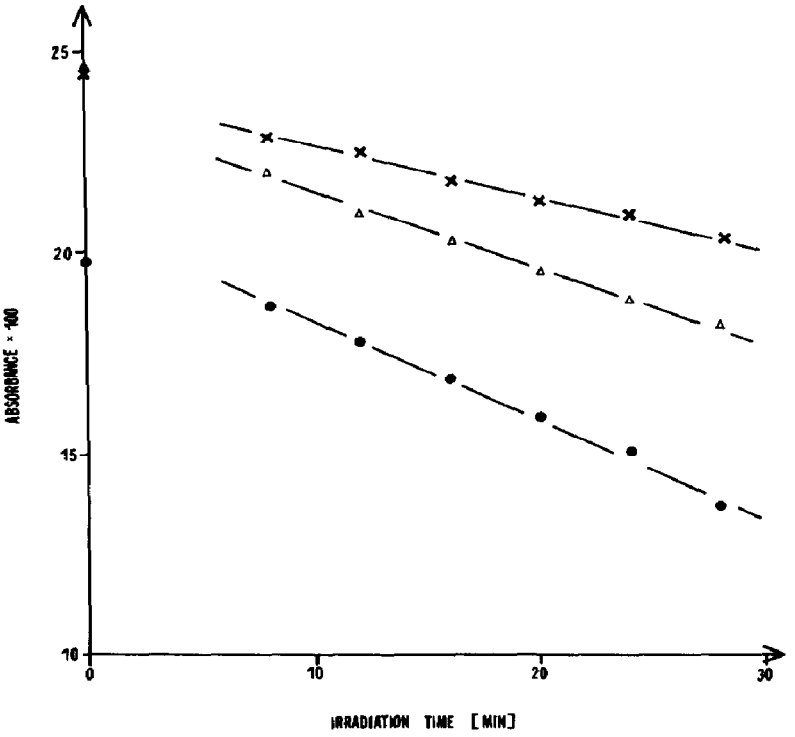

Fig. 1. Photobleaching of 1 in different aerated media (decrease in absorbance at 507 nm): ×, DTAB micelles; △, DPPC vesicles; ●, erythrocyte ghosts.

TABLE 1

Comparison of photobleaching of protoporphyrin IX in different homogeneous solutions and microheterogeneous media

Medium [a]	*Quantum yield for disappearance of 1* [b]
$CHCl_3$	1.0
Benzene	0.3
SDS micelles	0.8
DPPC vesicles	1.4
Erythrocyte ghosts	0.5

[a] Samples were irradiated with light of wavelengths longer than 366 nm in a merry-go-round apparatus in the presence of atmospheric oxygen.
[b] Quantum yields relative to those obtained in CH_2Cl_2.

A careful monitoring of the four visible peaks of 1 during the irradiation indicates that the rate of disappearance of the long wavelength peaks at 630 and 575 nm is somewhat faster than for the peaks at 541 and 505 nm. This suggests the formation of a product absorbing in the 500 - 550 nm range; however, several attempts to isolate or detect such a product have been until now unsuccessful.

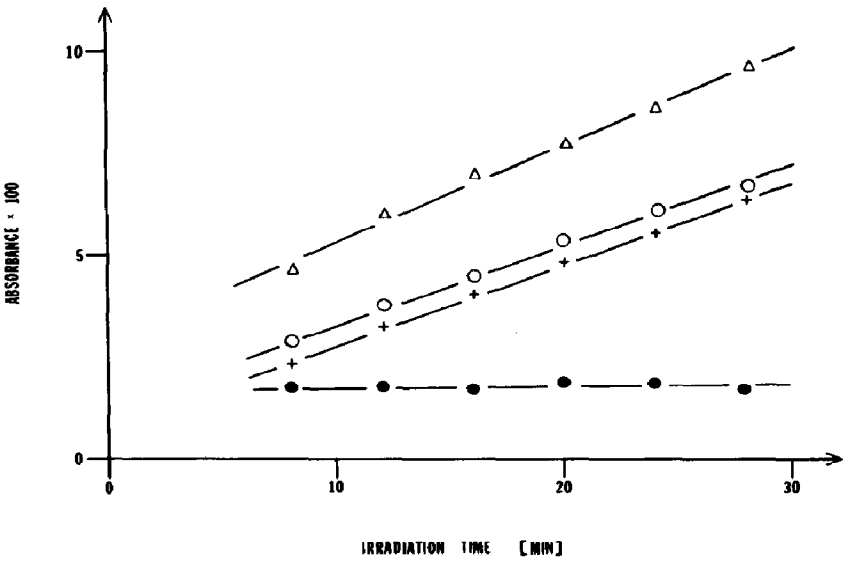

Fig. 2. Photogeneration of products 2 and 3 in different media (increase in absorbance at 670 nm): +, DTAB micelles; △, DPPC vesicles; ○, $CHCl_3$, ●, erythrocyte ghosts.

Irradiation of other free-base porphyrins such as mesoporphyrin IX, HPD (a mixture of hematoporphyrin IX (the major component), hematoporphyrin IX monoacetate and diacetate, 2,4-monovinyl-monohydroxyethyldeuteroporphyrin and 1), tetraphenylporphyrin and tetraphenylporphyrin zinc(II) was also carried out in aerated ghost solutions. For mesoporphyrin IX and HPD it was found that a photobleaching occurs at similar rates to that observed for 1 (Fig. 3). For both these porphyrin solutions no new visible-light-absorbing photoproduct could be detected. For tetraphenylporphyrin–ghost solutions irradiation produces very little degradation. In contrast, tetraphenylporphyrin zinc(II) in ghosts photoreacts to give a product absorbing strongly below 500 nm and in the 700 - 800 nm region; the photobehavior observed for this porphyrin in ghosts is thus very similar to that previously observed for zinc, cadmium and magnesium complexes of tetraphenylporphyrin in homogeneous solution in solvents such as CH_2Cl_2 [32, 33].

The results obtained with the various porphyrins on irradiation in aerated ghosts are clearly structure dependent; in several cases striking differences are observed compared with homogeneous solutions while in other cases the results are similar in the different media. Thus in summary 1 degrades slightly faster in ghosts than in homogeneous solution but it is clear that normal singlet oxygen photoproducts are not obtained. Mesoporphyrin IX and HPD degrade much more rapidly in ghosts than in homogeneous solution; the rapid reaction in the ghosts compared with a virtual unreactivity in organic solvents suggests a new mechanism may be involved. In contrast, tetraphenylporphyrin is unreactive in both media while tetraphenylporphyrin zinc(II) shows similar reactivity. The contrast between results in the

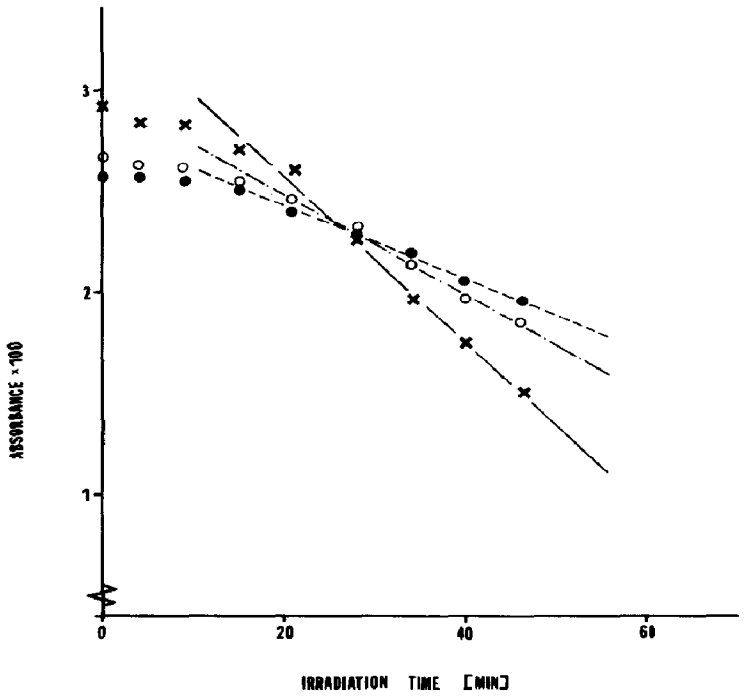

Fig. 3. Disappearance of different porphyrins by photobleaching in ghosts (decrease in absorbance at 500 - 510 nm): ×, mesoporphyrin IX; ●, HPD; ○, 1.

ghost solutions and microheterogeneous media such as micelles and phospholipid vesicles for several porphyrins suggests that the differences in reactivity are due perhaps more to specific components of the ghost cells rather than to an environmental effect provided by incorporation into a membrane or solubilization at a hydrophobic–hydrophilic interface. To investigate further the reasons for the differences between reactivity in media such as vesicles (or organic solvents) and the ghosts, we attempted to find a medium which would solubilize the porphyrins at an interfacial site of moderate polarity and which would simultaneously cosolubilize potential reactants which might be expected to play a role in photo-oxidation where singlet oxygen is produced.

3.2. Photo-oxidation studies in oil-in-water microemulsions

The medium chosen for these studies was a four-component microemulsion consisting of benzene, surfactant (anionic SDS or cationic DTAB), *n*-butanol and water, proportions increasing in the order listed. This clear microemulsion is clearly water rich and, on the basis of studies of similar media [34], should exist as small oil droplets, coated with the cosurfactants, in a bulk water phase. The several porphyrins studied above can all be solubilized in this medium and it is reasonable to anticipate that they would be solubilized at or near the droplet–water interface on the basis of other studies of moderately polar organic molecules in similar media [35, 36]. The

two microemulsions also have the advantage of solubilizing organic-insoluble species such as amino acids which are likely candidates for rapid reaction with singlet oxygen in ghosts and other natural membrane systems.

Irradiation of 1 in either the DTAB-based or the SDS-based microemulsions in the presence of oxygen leads to photo-oxidation of the porphyrin at rates approximately the same as those in homogeneous benzene solutions. Products 2 - 6 are produced in approximately the same ratio as in CH_2Cl_2. Thus it seems to be clear that reaction in the microemulsion alone is normal and involves singlet oxygen as the predominant reactive intermediate attacking ground state 1. Since one of the main differences between the ghosts and either the microemulsion or the phospholipid vesicles is the presence of protein components, it appeared likely that oxidizable amino acids present in the protein might be playing a role in the altered reactivity of 1, HPD and mesoporphyrin IX. Accordingly 1 was irradiated in both the DTAB and the SDS microemulsions in the presence of air and various amounts of amino acids such as histidine, cysteine, tryptophan, arginine and methionine. Results typical for 1 with the amino acids cysteine, histidine, tryptophan and methionine are shown in Figs. 4 and 5 for 1 with methionine in the DTAB microemulsion. As Fig. 4 indicates, addition of methionine in the range 10^{-2} - 10^{-3} M enhances the disappearance of 1; in contrast, adding increasing amounts of methionine suppresses the formation of 2 and 3 until at 1.3×10^{-2} M methionine the presence of the hydroxyaldehydes can scarcely be detected (Fig. 5). As was the case with irradiated ghost solutions containing 1, no new visible- or near-UV-light-absorbing products can be

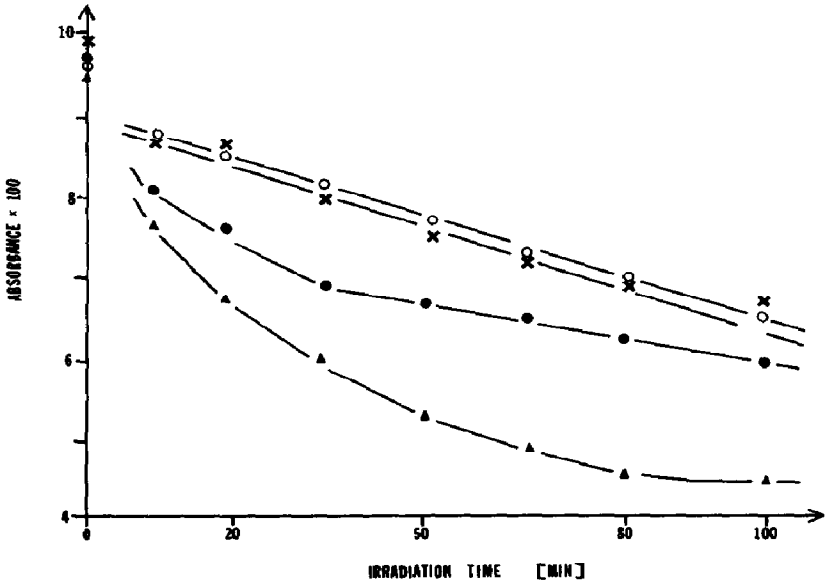

Fig. 4. Photobleaching of 1 in the DTAB microemulsion (decrease in absorbance at 634 nm) in the presence of different concentrations of methionine: ○, 0.0 M; ×, 1.3×10^{-3} M; ●, 3.3×10^{-3} M; ▲, 1.3×10^{-2} M.

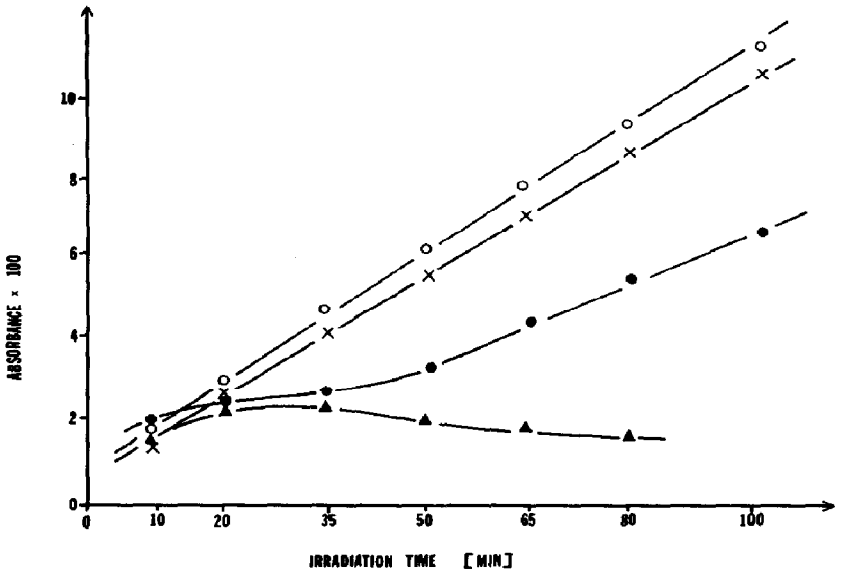

Fig. 5. Suppression of formation of 2 and 3 in DTAB microemulsions by addition of methionine (monitored at 670 nm): ○, 0 M; ×, 1.3×10^{-3} M; ●, 3.3×10^{-3} M; ▲, 1.3×10^{-2} M.

detected in the microemulsions with moderate concentrations of methionine and it appears to be clear that the net observable reaction is a general photodegradation of the porphyrin. As mentioned above, qualitatively similar results are obtained for cysteine, histidine, tryptophan and methionine; Figs. 6 and 7 compare the effects of several different amino acids on the disappearance of 1 and the appearance of 2 and 3. Amino acids such as arginine and norvaline (see below) were found to have almost no effect on the photodegradation of 1.

In contrast with the reactivity of 1 in aerated microemulsions alone, the octaalkyl porphyrins hematoporphyrin IX and mesoporphyrin IX which have no vinyl groups were found to be relatively photostable on irradiation in either the DTAB or the SDS microemulsions. Similarly, the HPD mixture is quite photostable in this medium. Addition of methionine to the microemulsions results in a bleaching of the porphyrin transitions for all three porphyrins hematoporphyrin IX, HPD and mesoporphyrin IX; Fig. 8 compares hematoporphyrin IX and mesoporphyrin IX with and without methionine. Here again no new visible- or near-UV-light-absorbing products can be detected concurrent with the photobleaching of the porphyrin bands. The similarity of bleaching rates for 1, hematoporphyrin IX and mesoporphyrin IX in the presence of methionine and other amino acids suggests a similar reaction path for all three porphyrins in ghosts and the microemulsion–amino acid solutions.† Interestingly it has been found that HPD, the reagent used in a number of exploratory cancer phototherapy treatments,

†See footnote to p. 239.

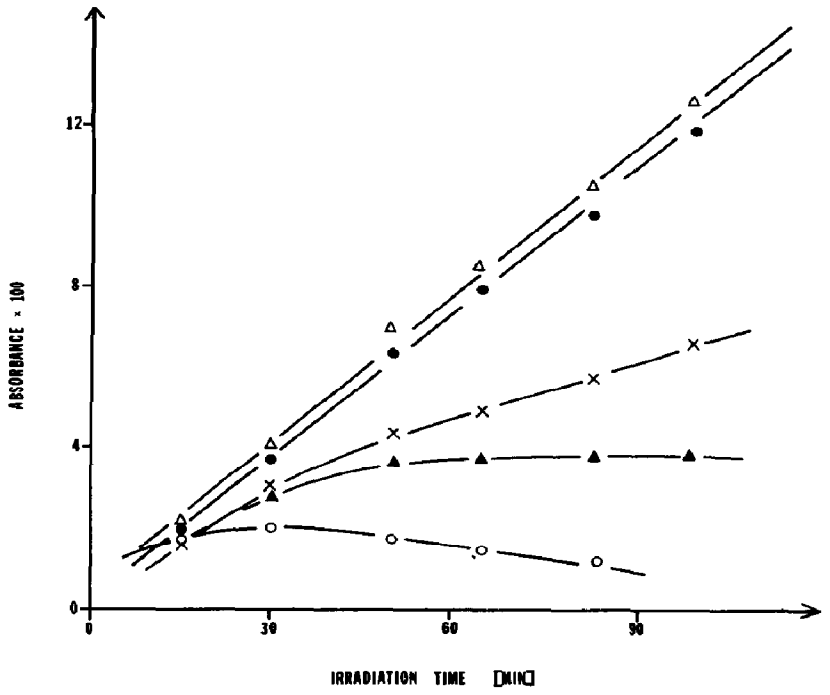

Fig. 6. Appearance of 2 and 3 in a DTAB microemulsion with added amino acids (concentration of amino acid, 1.3×10^{-2} M; wavelength, 670 nm): △, arginine; ●, water (no added amino acid); ×, cysteine; ▲, histidine; ○, methionine.

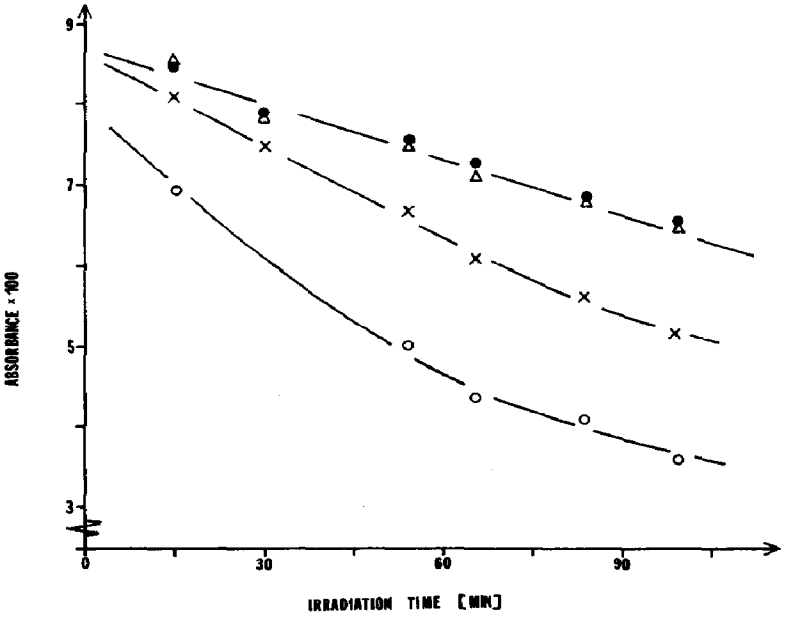

Fig. 7. Disappearance of 1 in DTAB microemulsion in the presence of amino acids (concentration of amino acid, 1.3×10^{-2} M; wavelength, 634 nm): △, arginine; ●, water (no added amino acid); ×, cysteine; ○, methionine.

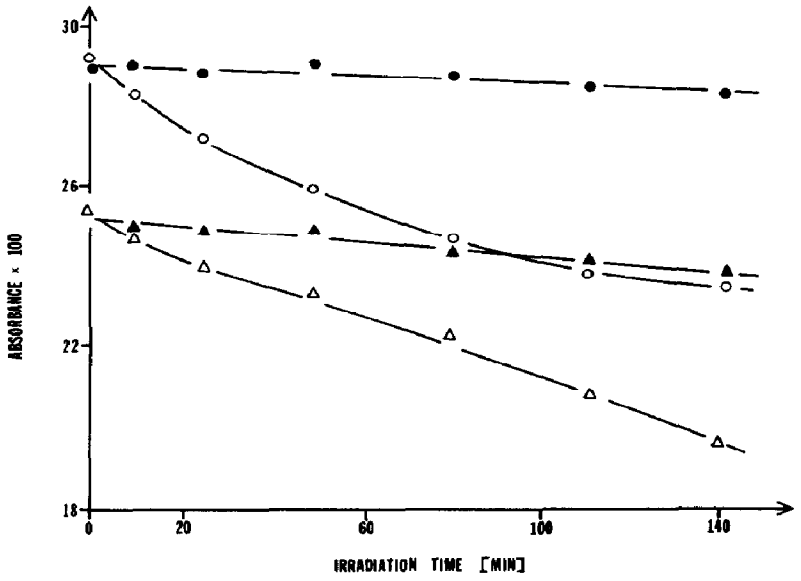

Fig. 8. Irradiation of hematoporphyrin IX and mesoporphyrin IX in the DTAB microemulsion with methionine (wavelength, 500 nm): ●, hematoporphyrin (no methionine); ○, hematoporphyrin plus 1.3×10^{-2} M methionine; ▲, mesoporphyrin (no methionine); △, mesoporphyrin plus 1.3×10^{-2} M methionine.

shows behavior qualitatively similar to 1, hematoporphyrin IX and mesoporphyrin IX in the DTAB microemulsions to which methionine has been added; however, its photobleaching is much slower.

Irradiation of tetraphenylporphyrin in the SDS or DTAB microemulsions results in little photobleaching of the porphyrin with or without added amino acids. In contrast, tetraphenylporphyrin zinc(II) undergoes its normal photobleaching reaction in the microemulsion alone; its photodegradation is *quenched* when methionine is added to the microemulsion and no new products are detected.

Returning to the reaction of the octaalkyl porphyrins in the presence of the various amino acids in the DTAB and SDS microemulsions, several experiments were carried out to delineate the role of methionine in its alteration of the photo-oxidation of 1. Figure 9 compares the disappearance of 1 in the presence of norvaline, methionine sulfoxide and diethyl sulfide. Norvaline, which possesses all the features of methionine except the thioether group, produces no effect and only the normal photo-oxidation of 1 is observed. Similarly, methionine sulfoxide, the preliminary oxidation product of methionine, is also inert at concentrations when methionine produces a strong effect. Methionine sulfoxide is found to be a coproduct of the oxidation of 1 in the microemulsion–methionine solutions; however, as discussed below, methionine sulfoxide should be a normal product formed by reaction of singlet oxygen with methionine. In contrast with the two amino acids, diethyl sulfide promotes an enhanced degradation of 1 qualitatively and quantitatively similar to that promoted by methionine, indicating that, for

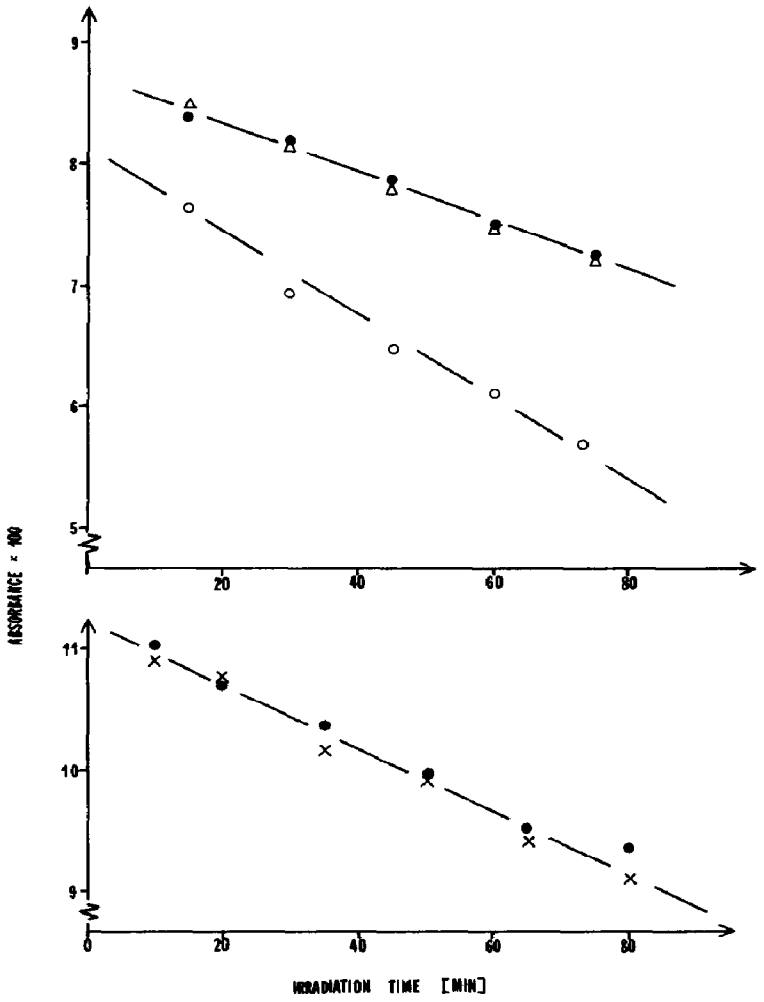

Fig. 9. Disappearance of 1 in DTAB microemulsion in the presence of various additives (concentration of additive, 1.3×10^{-2} M; wavelength, 634 nm): ●, water; △, norvaline; ○, diethyl sulfide; ×, methionine sulfoxide.

methionine, the thioether group is almost certainly the source of the altered photo-oxidation of 1 and the other octaalkyl porphyrins.

Finally the photo-oxidation of 1 was investigated in the DTAB microemulsion with and without methionine added in the presence of D_2O or H_2O. Previous studies have shown that reactions in which interception of singlet oxygen is competitive with its decay are subject to strong solvent isotope effects on changing from H_2O to D_2O. In the microemulsion without methionine the disappearance of 1 and the concurrent appearance of 2 and 3 are accelerated by D_2O replacement of water with $\phi_{D_2O,1}/\phi_{H_2O,1} = 2.7$. This appears to be a reasonable effect for a process involving singlet oxygen in an environment where there is somewhat reduced contact between 1 and singlet oxygen and the aqueous (D_2O) phase. In contrast, when 1 is irradiated in the

microemulsion in the presence of 0.013 M methionine, the photobleaching of 1 occurs with no detectable isotope effect.

4. Discussion

The results obtained on irradiation of porphyrins 1, hematoporphyrin IX, HPD and mesoporphyrin IX in erythrocyte ghosts and the microemulsions containing oxidizable amino acids strongly support a photo-oxidation process involving molecular oxygen but one which does not involve singlet oxygen as the reagent ultimately attacking the porphyrin. Evidence that singlet oxygen must be involved is easily obtained. The photodegradation of porphyrins 1, hematoporphyrin IX, HPD and mesoporphyrin IX does not occur in deaerated solutions. The fluorescence and triplet lifetime of 1 are not quenched in ghosts or the microemulsions in the absence of air. Addition of methionine (0.013 M) to the microemulsions containing 1 also causes no change in or quenching of the fluorescence or triplet–triplet absorption. Thus it seems to be quite clear that the porphyrin excited states are produced in these media and the primary photoprocess responsible for the observed photobleaching is quenching of porphyrin triplet states to yield singlet oxygen:

$$P \xrightarrow{h\nu} {}^1P^* \longrightarrow {}^3P^* \tag{2}$$

$${}^3P^* + O_2 \longrightarrow P + {}^1O_2^* \tag{3}$$

The normal photo-oxidation of 1 involves attack of singlet oxygen on the porphyrin

$$P + {}^1O_2^* \xrightarrow{k_p} 2 - 6 + 1 \tag{4}$$

to yield the various products 2 - 6. The rate constant k_p for this process is 8.3×10^5 M^{-1} s^{-1} [9]; this value is relatively low and comparable with those measured for several other porphyrins [37]. Consequently direct attack of singlet oxygen should be an important reaction only when no other reactive substrates are present. In the several homogeneous solutions previously studied as well as unadulterated micelles or vesicles made from fully saturated surfactants such a situation obtains and moderately efficient self-sensitized photo-oxidation of 1 to give 2 - 6 is the predominant path. However, in a natural membrane system such as the ghosts there are a host of potential substrates for singlet oxygen that would be expected to react with bimolecular rate constants greater than or equal to k_p. These include unsaturated lipids such as cholesterol ($k_s = 6.7 \times 10^4$ M^{-1} s^{-1}) [38] and a number of "oxidizable" amino acids such as those studied in the present investigation. That singlet oxygen is *rapidly* consumed is strongly implied by the lack of an H_2O–D_2O isotope effect on the rate of photobleaching in the microemulsions when methionine is present.

The present results strongly support a "new" photodegradation mechanism whereby the self-sensitized photo-oxidation of porphyrins 1, hematoporphyrin IX, HPD and mesoporphyrin IX involves a more complex sequence of reactions:

$$^1O_2^* + S \xrightarrow{k_s} \text{“}SO_2\text{”} \quad (5)$$

$$SO_2 \longrightarrow \text{substrate oxidation products} \quad (6)$$

$$SO_2 + P \longrightarrow P_{ox} + S \text{ or } S_{ox} \quad (7)$$

in which an easily oxidized substrate S is converted into a relatively good oxidant which can, in turn, in a ground state process oxidize the porphyrin. Although a number of candidates could be possible intermediates in such processes, the relatively higher rates of reaction towards singlet oxygen of reagents such as thioethers compared with simple unsaturated compounds [38] make the oxidizable amino acids attractive reagents for at least the initial studies.

The demonstration (see especially Figs. 4 and 5) that addition of several amino acids can suppress the formation of 2 - 6 while simultaneously enhancing the bleaching of 1 in the microemulsions suggests that protein components may well be playing a similar role in the ghost solutions. The fact that other octaalkyl porphyrins not containing vinyl groups are bleached at rates similar to 1 further suggests that reaction (7) must involve a direct attack on the porphyrin macrocycle and not at the side-chains.

While it is clear that a variety of intermediates could be formed from different amino acids that could subsequently attack the porphyrin macrocycle in dark or light processes, the experiments with methionine and some structurally related compounds rather clearly delineate at least one possible mechanism. First, our studies in the microemulsions show that while both methionine and diethyl sulfide mimic the effect of ghost solutions, norvaline, a non-sulfur-containing analog of methionine, has no effect on the normal self-sensitized photo-oxidation of 1. Furthermore, methionine sulfoxide, which is readily detected as a coproduct of the photobleaching of 1 in the microemulsions containing methionine, is also inert with respect to altering the self-sensitized photo-oxidation of 1. Thus it appears that the thioether group of methionine is essential for the new photobleaching and that oxidation of methionine to its sulfoxide is concurrent with oxidation of 1. Other studies have shown that thioethers are photo-oxidized to sulfoxides [39 - 41] and it has been suggested that persulfoxides can be involved as intermediates in these reactions. It seems then not unreasonable that for methionine a persulfoxide is the single oxygen donor in reaction (7) and that with other amino acids either hydroperoxides or perepoxides [42 - 46] could be reactive donors. More work clearly needs to be done in this area and current studies are under way to determine and detect intermediates involved with other amino acids and unsaturated lipids. However, the results with

methionine clearly indicate at least one specific path which could be important when singlet oxygen is generated in the vicinity of proteins.

The photobleaching of the porphyrins 1, hematoporphyrin IX, HPD and mesoporphyrin IX could involve a number of different reactions; however, the most likely path would appear to be one in which the porphyrin is epoxidized at a double bond between the ring and the methine bridge. Epoxides have previously been proposed (although in some cases apparently excluded) as intermediates in light-induced oxidation of metalloporphyrins to linear tetrapyrroles [32, 33]. Although we have been unable to detect any visible-light-absorbing products as a consequence of the porphyrin photobleaching, the differential bleaching of the visible bands of 1 in the ghosts and microemulsions suggests the possibility of intermediate products absorbing in the region where bilirubin and biliverdin pigments absorb [47]. These pigments are well known to degrade rapidly in the presence of singlet oxygen [47, 48].

The results obtained with tetraphenylporphyrin and tetraphenylporphyrin zinc(II) offer interesting contrasts with those for the octaalkyl porphyrins. The relative lack of reactivity of tetraphenylporphyrin towards both singlet oxygen and microemulsions containing the amino acids offers little substantial mechanistic insights although it could be suggested that the presence of a bulky phenyl substituent at the bridge carbon impedes attack of bulky oxygen donors such as a persulfoxide to the porphyrin macrocycle. The fact that the normal singlet oxygen photo-oxidation of tetraphenylporphyrin zinc(II) occurs in the ghosts and microemulsions alone, but not when methionine is added to the microemulsions, is surprising. The latter result suggests that interception of singlet oxygen by reaction (5) should avert oxidation of the zinc complex which would not be unreasonable in view of the lack of reactivity of tetraphenylporphyrin. However, the fact that tetraphenylporphyrin zinc(II) gives normal singlet oxygen products in the ghosts is unexpected in view of the results with 1 and hematoporphyrin IX, HPD and mesoporphyrin IX. A possible explanation may be that tetraphenylporphyrin zinc(II) resides in a different solubilization site in the ghosts than 1, hematoporphyrin IX, HPD and mesoporphyrin IX but this has certainly not been demonstrated. Additional experiments are currently in progress to determine the number and role of different solubilization sites in ghosts and other natural membranes and their role in photo-oxidation reactions and other processes.

Acknowledgment

We thank the U.S. Army Research Office for support of this research. M. Krieg thanks the Swiss National Science Foundation for a fellowship.

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