The effect on photohaemolysis of variation in the structure of the porphyrin photosensitizer

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A comparison of the photosensitizing ability of a variety of porphyrins for photohaemolysis gives the following order of activity: protoporphyrin > deuteroporphyrin, mesoporphyrin, haematoporphyrin dimethyl ester > haematoporphyrin diacetate, haematoporphyrin > haematoporphyrin monoacetate, coproporphyrin III, haematoporphyrin derivative, coproporphyrin III tetramethyl ester > uroporphyrin I, *meso*-tetra-(*N*-methyl-4-pyridinium)porphyrin tetratoluene-*p*-sulphonate, *meso*-tetra-(*p*-carboxyphenyl)porphyrin, protoporphyrin dimethyl ester, *meso*-tetra-(*p*-hydroxy-sulphonylphenyl)porphyrin tetrasodium salt, uroporphyrin III, deuteroporphyrin-3,8-disulphonic acid and protohaemin. The results for the metal-free porphyrins are rationalized in terms of solubility and partition properties, and a model is proposed for the incorporation of amphipathic porphyrins into the membrane lipid bilayer. Experiments with erythrocytes from patients with erythropoietic protoporphyria and with normal erythrocytes to which porphyrin was added in a deuterium oxide medium do not lead to an increase in the rate of photohaemolysis. A possible explanation for this somewhat surprising observation is outlined.

Photohaemolysis is a term used to describe the light-induced rupture of the erythrocyte membrane, and it is conveniently monitored by measuring the release of haemoglobin into the medium. It must be recognized that a number of changes precede the loss of a molecule as large as haemoglobin, but it is that change which is of interest in the present investigation. Various photosensitizers have been observed to promote photohaemolysis, but porphyrin photosensitization has been most widely studied, presumably because of the natural occurrence of porphyrin photosensitization of tissues in certain porphyrias.

Photohaemolysis was first observed in the early years of the century: the photosensitizing effect of eosin (von Tappeiner, 1908) and of porphyrins, including chlorophyll and haematoporphyrin (Hausmann, 1907, 1908), was recognized, as was the dependence on O₂ (Hasselbach, 1909). Renewed interest in the phenomenon arose in the mid 1960s especially with respect to porphyrin photosensi-

tization (Harber *et al.*, 1964; Fleischer *et al.*, 1966; Schothorst *et al.*, 1971; Hsu *et al.*, 1971). The porphyrins may be present naturally, as in erythrocytes containing protoporphyrin from patients with erythropoietic protoporphyria (EPP cells; Magnus *et al.*, 1961), or the protoporphyrin may be added to normal erythrocytes *in vitro* (pseudo-EPP cells). It seems reasonable to suppose that the naturally and artificially porphyrin-enriched cells are likely to differ with respect to the location in which the porphyrin finds itself. Nevertheless studies with pseudo-EPP cells have attracted considerable attention, and have potential relevance to the general question of the mechanism of photodamage by porphyrins. This has recently become of considerable importance in relation to tumour photonecrosis sensitized by porphyrins (Andreoni & Cubeddu, 1984). Porphyrins are known to photosensitize the formation of singlet oxygen (Foote, 1968), and this reactive species has been suggested to be responsible for photodamage (Lamola *et al.*, 1973). The evidence is not clear-cut, however, and other reactive intermediates, for example superoxide ion and other radical intermediates generated in a type I photo-oxidation

Abbreviations used: EPP, erythropoietic protoporphyria; TPPSS, *meso*-tetra-(*p*-hydroxy-sulphonylphenyl)porphyrin tetrasodium salt.

pathway (Schenck, 1963), are also possible (Grossweiner *et al.*, 1982). [It seems to us important to make a conceptual distinction between photo-processes in Nature that are evolved processes under metabolic control (such as photosynthesis and vision) and those that are not. For the latter, and photohaemolysis is such a process, it seems inherently unlikely that a single pathway will be followed in a system as complex as a cell (Bonnett *et al.*, 1984).] There is also some uncertainty about the membrane component that is the chief target. Some investigators have concluded that the major damage is caused by peroxidation of unsaturated fatty acid residues (Goldstein & Harber, 1972; Roshchupkin *et al.*, 1975) or of cholesterol (Lamola *et al.*, 1973; Bland *et al.*, 1975; Strom *et al.*, 1977), whereas others have considered the main pathway to involve cross-linking of membrane proteins (Girotti, 1976; de Goeij & van Steveninck, 1976; Dubbelman *et al.*, 1977, 1978; but see Lamola & Doleiden, 1980). In the present paper we examine the effect of structural variation in the porphyrin photosensitizer on the photohaemolysis of normal human erythrocytes. This amounts to carrying out the pseudo-EPP experiment but with porphyrins other than protoporphyrin. We have also examined the effect of using deuterated water as the suspension medium for both EPP cells and pseudo-EPP cells.

Materials and methods

Porphyrins

Protoporphyrin was prepared from protohaemin (Falk, 1964). Mesoporphyrin was purified as the dimethyl ester (Falk, 1964), which was then hydrolysed with 0.5% KOH in methanol/benzene (1:1, v/v). Deuteroporphyrin was obtained from protohaemin by a two-stage process involving fusion with resorcinol (Schumm, 1928) followed by demetallation with formic acid and iron powder (Ramsey, 1953; Falk, 1964). Haematoporphyrin and related compounds were prepared by Dr. P. A. Scourides as described by Bonnett *et al.* (1981). *meso*-Tetra-(*p*-hydroxysulphonylphenyl)porphyrin tetrasodium salt (TPPSS) was prepared by the method of Srivastava & Tsutsui (1973). *meso*-Tetra-(*p*-carboxyphenyl)porphyrin, *meso*-tetra-(*N*-methyl-4-pyridinium)porphyrin tetratoluene-*p*-sulphonate and deuteroporphyrin-3,8-disulphonic acid were gifts from Dr. R. J. Ridge. Coproporphyrin I and III were gifts from Professor A. H. Jackson and Professor A. R. Battersby. Uroporphyrin III was isolated from touraco feathers (Nicholas & Rimington, 1951).

All porphyrin solutions were prepared as required. The general procedure was to weigh out accurately a small amount (0.5–1 mg) of the solid por-

phyrin and, after any initial treatment to ensure complete dissolution, to make it up to a known volume with phosphate-buffered saline (see below). Protoporphyrin was dissolved in 2.7M-HCl and its concentration was determined spectroscopically ($\lambda_{\max}$, 408 nm, ϵ 262000 M⁻¹·cm⁻¹; Falk, 1964). The appropriate volume of the acidic solution was neutralized immediately before being added to the buffered suspension of erythrocytes. Solutions of mesoporphyrin, deuteroporphyrin, coproporphyrin III and its tetramethyl ester were made up in 0.1M-HCl and neutralized before use. Haematoporphyrin, haematoporphyrin derivative, uroporphyrins I and III and TPPSS were dissolved in phosphate-buffered saline and used as such. An initial trace of alkali was needed to dissolve protohaemin, haematoporphyrin acetates and the tetraphenylporphyrin derivatives before dilution with buffer. The solubilization of the haematoporphyrin acetates by alkaline treatment is known to cause chemical changes (elimination, hydrolysis, oligomerization; Berenbaum *et al.*, 1982). A solution containing photoporphyrins (Inhoffen *et al.*, 1969) was obtained by irradiating protoporphyrin (8–10 µg) in phosphate-buffered saline (50 ml) flushed with O₂ (see Table 2). The iso-osmotic buffer was prepared as follows. Solution A (34.99 g of Na₂HPO₄·12H₂O/l) and solution B (23.4 g of NaH₂PO₄·2H₂O/l) were made up. Then 100 ml of solution A and 19 ml of solution B were mixed to give a buffer solution, pH 7.4. Phosphate-buffered saline was obtained by mixing equal volumes of this buffer and 0.15M-NaCl (9 g/l).

Blood samples

Blood samples were obtained from healthy volunteers through the courtesy of Dr. D. Jenkins. Three samples of EPP cells were available, and had the following characteristics (porphyrin content for 100 ml of packed cells): sample 1, protoporphyrin (1370 µg) and coproporphyrin (10.5 µg); sample 2, protoporphyrin (776.8 µg); sample 3, protoporphyrin (352 µg). Porphyrin determinations were made by Mr. P. Fox by the method of Rimington (1971). For normal cells the protoporphyrin content per 100 ml of packed cells was approx. 30 µg.

Preparation of erythrocytes for photolysis

The blood was stored in heparinized tubes (1 mg of heparin/10 ml of blood) at 4°C and used within 3 days. Just before the experiment the blood was centrifuged (3000g for 5 min), and the buffy coat and plasma were discarded. The erythrocytes were washed thrice with phosphate-buffered saline at the centrifuge.

Typically for each experiment, 0.5 ml of washed

packed cells was suspended in 50 ml of phosphate-buffered saline. If appropriate, the suspension was treated with a known quantity of additive before making up to volume. The suspension was equilibrated at 15°C (1 h, in the dark) with gentle swirling. A control reaction was run in the absence of additive.

Photohaemolysis in deuterium oxide buffer

The general procedure (above) was varied to ensure maximum $^2\text{H}_2\text{O}$ exchange. The washed packed erythrocytes (1.5 ml) were suspended for 15 min in a slightly hyperosmotic solution containing 0.205 M-NaCl (12 g/l). The cells were separated at the centrifuge and suspended in iso-osmotic phosphate-buffered saline made in $^2\text{H}_2\text{O}$ (50 ml) to give 1% packed volume as before.

Photohaemolysis reactions

These were carried out in two identical water-cooled cylindrical Pyrex vessels (2.5 cm deep $\times$ 7 cm diam., approx. 100 ml) operated at $15 \pm 0.5^\circ\text{C}$. O_2 (or N_2) was passed at 55 ml/min through a sintered-glass distributor that was 3 cm below the surface. The suspension was subjected to gentle magnetic stirring. The light source was a 12 V 75 W tungsten filament (Lucas sealed beam unit 542-23-449), the centre of the outer glass surface of which was 10 cm from the vertical plane surface of the reactor vessel. The irradiance 10 cm from this lamp along the axis was found to be 13.1 mW/cm^2 ($80 \times$ Optometer; United Detector Technology). A control experiment, without an additive, but in the presence of O_2 and light, was performed each day. (Control experiments were also performed in the dark and under N_2 .) The haemolysis was determined at intervals of 30 min. by withdrawing a sample (1 ml), which was immediately centrifuged, the supernatant (0.5 ml) being diluted to 5.0 ml with Drabkin's solution. After 15 min the absorbance was measured at 540 nm (Drabkin & Austin, 1935). The percentage photohaemolysis due to added photosensitizer is defined as $100(A_1 - A_2)/A_x$, where, for a given time, A_1 is the absorbance for the test sample and A_2 is the absorbance for the control (i.e. without additive); A_x is the absorbance for total haemolysis, which was determined after lysis of 0.5 ml of packed cells in 50 ml of distilled water. Percentage haemolysis is defined as $100A_1/A_x$. The estimated error is $\pm 5\%$.

Results

Control experiments

Experiments were conducted to establish the effect of the reaction conditions on autohaemolysis (in the absence of light) and 'autophotohaemolysis' (in the presence of light and O_2). About 3% of auto-

haemolysis was observed after 3 h, and about 7% of 'autophotohaemolysis' was observed in this system in that time. The increment presumably arises, in part, from the protoporphyrin present in normal erythrocytes at low concentrations. The effects of light, added protoporphyrin and O_2 were separately examined over a 3 h period. Some of the effects were not insignificant (e.g. up to 25% haemolysis after 3 h with N_2 , light and $0.574 \mu\text{M}$ -protoporphyrin, possibly owing to inevitable traces of O_2 in the system), but in general marked haemolysis was observed only when O_2 , light and protoporphyrin were employed together. Fig. 1 shows the effects of porphyrin concentration on haemolysis for the pseudo-EPP system, and for comparison, Fig. 2 shows results with erythrocytes

Fig. 1. Haemolysis of pseudo-EPP cells — — —, $1.016 \mu\text{M}$ -Protoporphyrin, light, O_2 ; —, $0.56 \mu\text{M}$ -protoporphyrin, light, O_2 ; ····, $0.622 \mu\text{M}$ -protoporphyrin, light, N_2 . For details see the Materials and methods section.

Fig. 2. Haemolysis of EPP cells ○—○, Patient 1: protoporphyrin, $1370 \mu\text{g}/100 \text{ ml}$ of packed cells, and coproporphyrin, $10.5 \mu\text{g}/100 \text{ ml}$ of packed cells; light, O_2 . ○—○, Patient 2, protoporphyrin, $776.8 \mu\text{g}/100 \text{ ml}$ of packed cells; light, O_2 . ○—○, Patient 3, protoporphyrin, $352 \mu\text{g}/100 \text{ ml}$ of packed cells; light, O_2 . ●—●, Patient 1: as before; light, N_2 . For details see the Materials and methods section.

Table 1. *Photohaemolysis of erythrocytes sensitized with various porphyrins*
For details see the Materials and methods section.

Porphyrin	Molarity (μM)	Photohaemolysis after 3 h (%)
Protoporphyrin (1)	0.37	81
	0.56	93
	1.02	94
Deuteroporphyrin (2)	0.56	78
	0.68	95
Mesoporphyrin (3)	0.56	75
	0.62	85
Haematoporphyrin dimethyl ester	0.61	70
	0.76	86
Haematoporphyrin diacetate (4)	0.46	48
	0.76	70
Haematoporphyrin (5)	0.84	22
	1.26	23
Haematoporphyrin monoacetate (6)	0.45	15
	0.76	51
Coproporphyrin III (7)	0.56	12
	0.76	37
Haematoporphyrin derivative	0.72	11
	0.96	33
Coproporphyrin III tetramethyl ester	0.56	5
	0.76	19
Uroporphyrin I (8)	0.58	9
	0.77	10
	1.15	16
<i>meso</i> -Tetra-(<i>N</i> -methyl-4-pyridinium)porphyrin tetratoluene- <i>p</i> -sulphonate (9)	0.80	6
	1.92	13
<i>meso</i> -Tetra-(<i>p</i> -carboxyphenyl)porphyrin (10)	1.58	8
	3.01	14
Protoporphyrin dimethyl ester	0.70	5
	1.41	6
<i>meso</i> -Tetra-(<i>p</i> -hydroxysulphonylphenyl)porphyrin tetrasodium salt (11)	0.78	6
	1.92	13
Uroporphyrin III (12)	0.54	0
	2.70	5
Deuteroporphyrin-3,8-disulphonic acid (13)	0.50	0
	0.67	11
Protohaemin (14)	0.74	1
	0.92	1
	1.60	2

- (1) $\text{R} = \text{R}' = \text{CH}=\text{CH}_2$
 (2) $\text{R} = \text{R}' = \text{H}$
 (3) $\text{R} = \text{R}' = \text{Et}$
 (4) $\text{R} = \text{R}' = \text{CH}(\text{OAc})\text{CH}_3$
 (5) $\text{R} = \text{R}' = \text{CH}(\text{OH})\text{CH}_3$
 (6) $\text{R} (\text{R}') = \text{CH}(\text{OH})\text{CH}_3$
 $\text{R}' (\text{R}) = \text{CH}(\text{OAc})\text{CH}_3$
 (13) $\text{R} = \text{R}' = \text{SO}_3\text{H}$

from three EPP patients. Both figures show control curves for experiments under N_2 that confirm the involvement of O_2 in the photohaemolytic process.

Photohaemolysis: effect of porphyrin structure

Photohaemolysis sensitized by protoporphyrin shows a marked induction period, followed by a rapid reaction. Since the other porphyrins studied here show this behaviour less clearly or not at all, the only effective way of presenting the results is graphically. This is done in Figs. 3 and 4 for some of the porphyrins studied in the concentration range $0.50\text{--}0.80\mu\text{M}$. In Table 1 the amounts of photohaemolysis due to additive at an arbitrary time (3 h) are compared for all the porphyrins studied. This serves to rank the substances in a general way. Although it must be observed that the numerical values for the lower activities are subject to a large experimental error, the experiments have been repeated by three workers independently with essentially the same result as far as general classification (see the Discussion section) is concerned.

The results for experiments with photoprotoporphyrin preparations (i.e. pre-irradiation experiments) are shown in Table 2. For an irradiation in methanol, the amount of photoprotoporphyrin generated after 23 h was about 10% under the conditions employed, as estimated from the absorb-

ance of the new peak at 668 nm, and assuming the same molar absorption coefficient as that reported (λ_{max} , 668 nm, ϵ 57000 $\text{M}^{-1}\cdot\text{cm}^{-1}$) for the diester of isomer A in this solvent (Inhoffen *et al.*, 1969). In

Fig. 3. Haemolysis of erythrocyte suspension under standard conditions in the presence of O_2 and light with the following porphyrin additives: protoporphyrin ($0.56\mu\text{M}$; $\circ\text{---}\circ$), deuterioporphyrin ($0.56\mu\text{M}$; $\circ\cdots\circ$), mesoporphyrin ($0.55\mu\text{M}$; $\circ\cdots\circ$), meso-tetra-(*p*-carboxyphenyl)porphyrin ($0.80\mu\text{M}$; $\bullet\text{---}\bullet$) and TPPSS ($0.78\mu\text{M}$; $\bullet\cdots\bullet$).

For standard conditions see the Materials and methods section.

Table 1—cont.

(7) $P = \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$

(8) $A = \text{CH}_2\text{CO}_2\text{H}$

(12)

(14)

Table 2. Photohaemolysis of pseudo-EPP erythrocytes prepared with protoporphyrin (160–362 nm) with and without pre-irradiation of sensitizer in buffer

This set of experiments was carried out by a different investigator and under somewhat different conditions. Light-source: Philips Argaphoto B 500W. Buffer: 26 ml of 0.2M- NaH_2PO_4 + 174 ml of 0.2M- Na_2HPO_4 + 4 g of NaCl was made up to 400 ml (pH 7.4).

Expt. no.	Duration of pre-irradiation (h)	Photohaemolysis at time indicated		Photohaemolysis in control (i.e. sensitizer not pre-irradiated) at same time (%)
		Time (h)	(%)	
1	5	4	51	85
2	6	3	23	86
		4	45	89
3	10.5	1.75	24	91
		2.0	33	92
		2.5	71	91
4	17.0	1.5	16	76
		1.75	38	88
		2.0	62	92

Fig. 4. Haemolysis of erythrocyte suspensions under standard conditions in the presence of O_2 and light with the following porphyrin additives: protoporphyrin ($0.56 \mu\text{M}$; $\bigcirc$ — $\bigcirc$), uroporphyrin I ($0.58 \mu\text{M}$; $\bigcirc$ --- $\bigcirc$), coproporphyrin III ($0.56 \mu\text{M}$; $\bigcirc$... $\bigcirc$) and uroporphyrin III ($0.54 \mu\text{M}$; $\bullet$ — $\bullet$).

For standard conditions see the Materials and methods section.

all cases pre-irradiation diminished the extent of haemolysis.

Photohaemolysis with protoporphyrin in deuterium oxide

The photohaemolysis experiment was carried out in phosphate-buffered saline made up in $^2\text{H}_2\text{O}$ with EPP cells and with pseudo-EPP cells. For EPP cells (three separate examples) virtually no effect was observed (a slight decrease in photohaemolysis was observed with cells from one weakly porphyric patient). For pseudo-EPP cells there was a marked decrease in the initial rate of photohaemolysis, as shown in Fig. 5.

Fig. 5. Haemolysis of pseudo-EPP cells ($0.75 \mu\text{M}$ -protoporphyrin) in $^1\text{H}_2\text{O}$ buffer (—) and in $^2\text{H}_2\text{O}$ buffer (---).

For details see the Materials and methods section.

Discussion

Although the differences in the shapes of the photohaemolysis curves make precise comparisons difficult, it is evident that the effectiveness of the porphyrins studied here as sensitizers falls broadly into five categories: A, very high activity ($>90\%$ photohaemolysis after 3 h at approx. $0.50 \mu\text{M}$), protoporphyrin (1); B, high-activity (70 – 90% photohaemolysis), deuteroporphyrin (2), mesoporphyrin (2), mesoporphyrin (3) and haematoporphyrin dimethyl ester (5 diester); C, intermediate activity (20 – 70% photohaemolysis), haematoporphyrin diacetate (4) and haematoporphyrin (5); D, low activity (10 – 20% photohaemolysis), haematoporphyrin monoacetate (6), coproporphyrin III (7), haematoporphyrin derivative and coproporphyrin III tetra-

methyl ester (7 tetraester); E, very low or zero activity ($<10\%$ photohaemolysis), uroporphyrin I (8), *meso*-tetra-(*N*-methyl-4-pyridinium)porphyrin tetratoluene-*p*-sulphonate (9), *meso*-tetra-(*p*-carboxyphenyl)porphyrin (10), protoporphyrin dimethyl ester (1 diester), *meso*-tetra-(*p*-hydroxy-sulphonylphenyl)porphyrin tetrasodium salt (TPPSS) (11), uroporphyrin III (12), deuteroporphyrin-3,8-disulphonic acid (13) and protohaemin (14).

These divisions are, of course, arbitrary. The last two categories are not well differentiated, and the numbers within them are particularly subject to error, the amount of haemolysis being inevitably only marginally greater than the control values. Nevertheless the broad distinctions arrived at in this way are useful: such a differentiation between the best-known porphyria porphyrins, that is between protoporphyrin on the one hand and coproporphyrin on the other, has been noted previously by Haining *et al.* (1969), but the present results considerably extend the earlier observation.

It is conceivable that the differences observed in the photohaemolytic activity of the various porphyrins could be associated with differences in their photophysical parameters. This is certainly the case for protohaemin: the excited states there are very short-lived (Adar *et al.*, 1976), and everyday experience shows that protohaem (or rather the protohaem of haemoglobin) is not a photosensitizer for singlet-oxygen formation. As far as absorption of light energy is concerned, the metal-free porphyrins studied here are, with the exception of photoporphyrin, expected to be rather similar, since they all possess a strong Soret band, and in the visible region are all characterized by absorption spectra of the etio type. Although differences in molar absorption exist, the major distinctions in photohaemolytic activity cannot be due to this cause. Differences in triplet properties occur, but again are not marked. The triplet energies of a number of porphyrins are known from phosphorescence spectra (Gouterman & Khalil, 1974) and are generally rather similar to one another (140–156 kJ/mol). For a range of porphyrin carboxylic acid methyl esters in benzene the triplet quantum yields (ϕ_T 0.63–0.88), the rate constants for triplet decay (3.7×10^3 – 4.8×10^3 s $^{-1}$) and the rate constants of triplet quenching by molecular O $_2$ (1.4×10^3 – 2.7×10^9 M $^{-1}$ ·s $^{-1}$) fall into quite narrow ranges (Bonnett *et al.*, 1980).

Although it may emerge that significant differences exist for the photophysical properties of the monomeric free acids, it seems more likely on present evidence that the sensitizing activity of these compounds in photohaemolysis may be principally related to their solubility, aggregation and partition characteristics. Those porphyrins that are

more-or-less soluble in water, such as uroporphyrin, coproporphyrin and the derivatives of tetraphenylporphyrin in Table 1, are presumably too polar to become effectively incorporated into the erythrocyte membrane under the conditions studied in the present work, and the porphyrins that are essentially insoluble in water, such as protoporphyrin dimethyl ester, presumably form aggregates in the aqueous medium, so that again the likelihood of incorporation is diminished. In terms of partition coefficients between phosphate-buffered saline and octan-2-ol (Kessel, 1977), compounds with very high or very low values appear to be ineffective. What seems to be required is a porphyrin with an intermediate partition coefficient value, and a structure that is amphipathic. This sort of structure is found in protoporphyrin, *meso*-porphyrin and deuteroporphyrin, where the alkylated porphyrin nucleus is lipophilic and the carboxylate anions hydrophilic, and, less obviously, in haematoporphyrin dimethyl ester (where the amphipathicity is reversed, and the two alcohol groups may fulfil the hydrophilic function). An extension of this idea leads to a model for the incorporation of, say, protoporphyrin into the lipid bilayer membrane as shown in Fig. 6. Support for this interpretation comes from the pioneering work of Stenhagen & Rideal (1939) on the behaviour of porphyrins towards monolayers in the Langmuir trough. These experiments showed that protoporphyrin readily penetrated into a lipid film, whereas haematoporphyrin and coproporphyrin were much less effective. Work by Emiliani & Delmelle (1983) on the effectiveness of various porphyrins on the photo-oxygenation of cholesterol embedded in egg-lecithin liposomes provides further support in a different model. In this system, the activities of the photosensitizers, as judged by the production of the 5 α -hydroperoxide from cholesterol, are deuteroporphyrin > protoporphyrin > haematoporphyrin $\gg$ uroporphyrin, which sequence is clearly similar to that observed in the present work with the erythrocyte system.

Haematoporphyrin and its mono- and di-acetates fall in the middle ranges of activity. Haematoporphyrin derivative, which is made by treating haematoporphyrin with H $_2$ SO $_4$ in acetic acid, consists mainly of a mixture of these acetates (Bonnett *et al.*, 1981), and has attracted attention because, on treatment with alkali, it is an effective sensitizer of tumour photonecrosis (Dougherty *et al.*, 1978; Berenbaum *et al.*, 1982). Haematoporphyrin derivative has only modest activity in photohaemolysis, with or without alkali treatment. Thus the photohaemolysis experiment is not a satisfactory model for tumour photosensitization. This is confirmed by the result with TPPSS: this has very low activity in photohaemolysis, but is an effective

Fig. 6. Schematic representation of the proposed intercalation of protoporphyrin into the lipid bilayer portion of the erythrocyte membrane

The lipid represented is phosphatidylcholine, which is a major component of human erythrocyte phospholipids (Cornwell *et al.*, 1968); other lipids (e.g. cholesterol, glycolipids) and associated protein are not shown. The heavy bar represents the intercalated porphyrin ring in side view. The bilayer is represented at high O_2 partial pressure (as in our experiments) to illustrate an explanation of the observations in 2H_2O (see the text).

Scheme 1

photosensitizer for tumour sensitization (M. C. Berenbaum, personal communication).

It seemed possible that the very high activity of protoporphyrin might be associated with the known photo-oxygenation reaction at the vinyl functions (Inhoffen *et al.*, 1969, experiment with

the dimethyl esters), which produces the photoprotoporphyrins A and B (Scheme 1). Indeed, the dimethyl esters of photoprotoporphyrins A and B have been shown to be effective sensitizers of singlet-oxygen formation (Cox *et al.*, 1982). Here the dicarboxylic acids (15) and (16) were needed

and, because of the instability of the individual photoporphyrin dimethyl esters under hydrolytic conditions designed to generate the free dicarboxylic acids, the experiment to test this postulate was conducted in an indirect way. The photoporphyrin free acids were generated in aqueous buffer (or in methanol) by pre-irradiation of protoporphyrin in the presence of O_2 . However, the buffer containing the photoporphyrin isomers was always less effective as a photosensitizer than was protoporphyrin itself. The reaction now showed a longer induction period, although the extent of haemolysis eventually reached was about the same as in the control without pre-irradiation (Table 2). Since absorbance measurements suggest that there is relatively little photodestruction of the porphyrin chromophore during the pre-irradiation period, it is concluded that the photoporphyrin isomers are less effective photosensitizers than is protoporphyrin under these conditions.

Finally, we turn to a result that initially caused some surprise. If the photohaemolysis proceeds by a singlet-oxygen mechanism, as has been commonly suggested (e.g. Lamola *et al.*, 1973), it would be expected to be faster in 2H_2O than in 1H_2O because the lifetime of singlet oxygen is greater in the former solvent (Merkel & Kearns, 1972; Kajiwarra & Kearns, 1973). It has, indeed, been shown that, after a 30 min exposure to near-u.v. irradiation (350–380 nm), photohaemolysis sensitized by haematoporphyrin proceeded more extensively in 2H_2O than in 1H_2O (Nilsson *et al.*, 1975). We have carried out experiments in 2H_2O with EPP cells and pseudo-EPP cells. With the former there is virtually no difference between the two solvent systems. With pseudo-EPP cells, photohaemolysis actually proceeds more slowly in 2H_2O than in 1H_2O , as shown in Fig. 5.

That the results are different for EPP and pseudo-EPP cells presumably relates to differences in concentration and location of protoporphyrin in the two types of cells. However, the lack of a rate increase should not be taken as an argument against a singlet-oxygen mechanism, for the following reasons. The solubility of O_2 in water is rather low (Bunsen absorption coefficient 0.0310 at 20°C), but it is higher in organic solvents (e.g. diethyl ether, Bunsen absorption coefficient 0.415 at 20°C; Landholt-Bornstein, 1923, 1931). Hence the concentration of O_2 within the lipid bilayer of a membrane is expected to be relatively high in the O_2 saturated system. Conversely, the concentration of water in this hydrophobic part of the bilayer is expected to be relatively low. We have referred above to the incorporation of protoporphyrin into the lipid bilayer and have envisaged that amphipathic interactions would generate an oriented

structure, as shown schematically in Fig. 6. Hence the chromophore of the protoporphyrin sensitizer and the O_2 are expected to be present in the hydrophobic region, along with the molecular species (e.g. unsaturated lipid, steroid) that is capable of reacting with singlet oxygen. If this model is correct, the presence of 2H_2O in the hydrophilic region would be expected to have little or no effect on the lifetime of the singlet oxygen generated within the membrane. The diminution of rate with pseudo-EPP cells (Fig. 5) is evidently associated with changes in the induction period, the chemical basis of which remains to be unravelled.

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