A New Method to Detect Porphyrin Radicals in Aqueous Solutions

J. MOAN

Biophysics Department, Norsk Hydro's Institute for Cancer Research, Montebello, Oslo 3, Norway

The stable free radical 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TAN) is an efficient scavenger of porphyrin anion radicals. The reaction between the two radicals leads to elimination of the spin of TAN. This reaction is convenient for detection of porphyrin radicals as well as for determinations of quantum yields. Total scavenging of photo-produced anion radicals of hematoporphyrin was observed at TAN concentrations above 1 mM.

The porphyrins are of considerable interest in photochemistry and photobiology because of their efficiency as photosensitizers. It has been proposed that this property may be used in phototherapy of cancer,[1−3] and the most recent clinical results seem to be very promising.[4] In aerated solution the porphyrins seem to act mainly by the singlet oxygen mechanism.[5,6]

However, recently it was proposed that porphyrin radicals might play a significant role in the photohemolysis of red cells of patients suffering from porphyrias.[7] Such radicals may play an even greater role in the porphyrin sensitized photoinactivation of cells in a tumor, since their yield is expected to be much higher under anoxic conditions, such as in a tumor, than under aerated conditions. Porphyrin radicals have mainly been studied by means of flash photolysis. We here propose an alternative method, which is convenient for measurements of reactions and yields of porphyrin anion radicals. The method is based on the fact that porphyrin anion radicals react with 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TAN). The reaction leads to an elimination of the spin of TAN.

MATERIALS AND METHODS

2,2,6,6-Tetramethyl-4-piperidone-N-oxyl, TAN, was prepared according to the method of Rozantsev.[8] Hematoporphyrin free base (HP) from Sigma was dissolved in 20 mM NaOH and brought to pH 6.8 by addition of 1 M HCl. Hydroquinone and pyrogallol (DAB 6) were obtained from Matheson, Coleman & Bell and Merck, respectively. All solutions were buffered to pH 6.8 with phosphate buffer, final concentration 20 mM. The light source was an Osram HBO 200 W high pressure mercury lamp fitted to a Bausch and Lomb grating monochromator. The sample solution (1.5 ml) was exposed to monochromatic light with a wavelength of 405 nm and an intensity of 3 mW/cm^2, as measured with a calibrated thermopile (Radiometer, model 65A, Yellow Springs Instruments, Ohio). The ESR spectrometer was an X-band instrument built in our institute with 110 kHz field modulation and a TE$_{102}$ cavity. The sample holder was a quartz cuvette of dimensions $20 \times 7 \times 0.5$ mm. ESR measurements were carried out at room temperature.

RESULTS

Fig. 1 shows that TAN in hypoxic solutions was degraded when exposed to light in the presence of HP. No significant degradation was observed in solutions saturated with air or oxygen (Fig. 1). Furthermore, no degradation was observed when anoxic or oxic solutions of TAN were exposed to HP in the darkness or to light in the absence of HP.

In addition to O_2 we studied the effect of the following scavengers of triplet HP:[9] $K_4Fe(CN)_6$, $K_3Fe(CN)_6$ and NaI. All these compounds reduced the HP sensitized photodegradation of TAN in anoxic solutions.

The effect of the following reducing agents was studied: pyrogallol, sodium ascorbate and
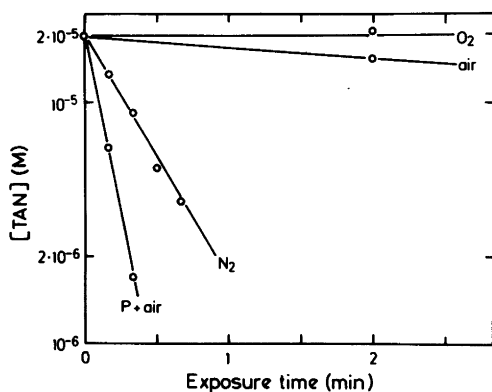
Fig. 1. Irradiation of 20 μM 2,2,6,6-tetramethyl-4-piperidone-N-oxyl, TAN, in aqueous solution (pH 6.8, 10 mM phosphate buffer) in the presence of 0.1 mM HP. The samples were flushed with N_2, O_2 or air during irradiation. The curve marked P+air corresponds to a sample irradiated in the presence of 10 mM pyrogallol.

glutathione. These compounds are known to donate electrons to HP molecules in the triplet state and thus enhance the formation of anion radicals of HP (HP⁻).[9,10] We found that these compounds strongly enhanced the HP sensitized photodegradation of TAN in hypoxic solutions. Pyrogallol and glutathione in concentrations up to 0.1 M had no effect on TAN in the darkness when studied on a timescale relevant to the present experiments, *i.e.*, up to 10 min. Neither did these compounds sensitize any photodegradation of TAN in the absence of HP. Ascorbate caused a slow degradation of TAN in the darkness as well as in light. This degradation rate, however, was only a few percent of the degradation rate during exposure to light in the presence of HP.

Pyrogallol was the most efficient of the three compounds in enhancing the HP sensitized photodegradation of TAN. Therefore we studied its
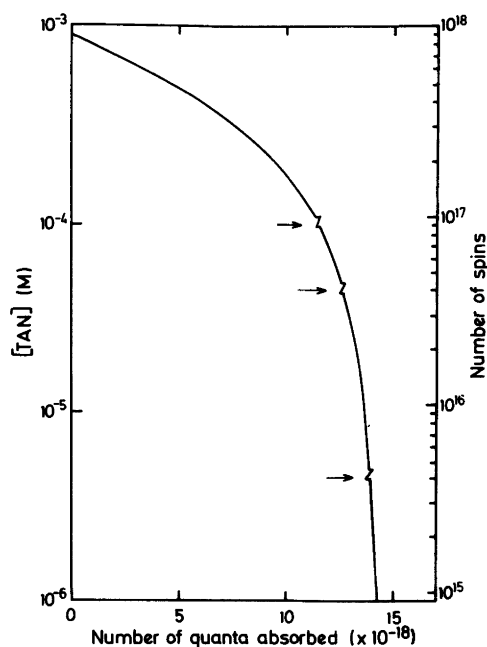

Fig. 2. Irradiation of TAN in aqueous solutions (aerobic conditions) in the presence of HP and 10 mM pyrogallol. HP is also slowly degraded during the irradiation. Therefore, the curve is composed of the data from four independent runs with different initial concentrations of TAN as indicated by the arrows.

effects somewhat in detail. TAN was efficiently photodegraded even in aerated solutions when HP and pyrogallol was present (Fig. 2). From curves like Fig. 1 the quantum yield of TAN degradation may be determined (Table 1). This quantum yield increased with increasing concentration of TAN and reached a plateau at about 1 mM TAN. In the presence of 20 mM pyrogallol or more the yield of TAN degradation was essentially the same in aerated and hypoxic solutions.

Table 1. Quantum yields for the degradation of TAN during irradiation in the presence of HP and pyrogallol.

TAN/mM	Pyrogallol/mM	Gassing	Quantum yield
5×10^{-3}	10	Air	0.013
1	10	Air	0.09
1	10	N_2	0.20
1	35	Air	0.25
1	35	N_2	0.26

DISCUSSION

Flash photolysis experiments have shown that when solutions of HP are exposed to light, triplet states of HP and anion radicals, HP^-, are formed.[9] Furthermore, singlet oxygen is formed in aerated solutions.[5,6] The photodegradation of TAN in the presence of HP is not mediated by singlet oxygen since it is eliminated in oxygenated solutions (Fig. 1). Neither is the degradation caused by HP molecules in the triplet state since well-known quenchers of HP triplets[9] such as pyrogallol and sodium ascorbate does not reduce or eliminate the degradation. We assume that the degradation of TAN is caused by a reaction between TAN and anion radicals, HP^-. This assumption is supported by the observation that electron donors such as pyrogallol, sodium ascorbate and glutathione enhance the degradation. Other investigators have shown that these agents enhance the yield of photoinduced HP^- radicals by donating electrons to HP molecules in the triplet state.[9,10] We therefore propose that TAN may be used as a probe for photo-produced HP^- radicals in aqueous HP solutions. The present work indicates that 1 mM TAN is sufficient to scavenge all the HP^- radicals formed in an aqueous solution. Thus, this concentration of TAN may be used to estimate the quantum yield of HP^- radicals. Table 1 shows quantum yields determined under different conditions. It can be seen that in solutions where the pyrogallol concentrations are high the yields are almost identical in aerobic and anaerobic solutions, as one would expect, since oxygen and pyrogallol compete for the excited HP molecules. Furthermore, the maximum yield of HP^- is obviously as high as about 0.26 (Table 1). For comparison the yield of $^1O_2$ is 0.75 in ethanol solutions of HP.[11] Thus, under aerobic conditions the main fraction of the triplet states of HP gives rise to $^1O_2$ if no triplet quencher or reducing agent is present. If a reducing agent such as pyrogallol is present a large fraction of the triplet states gives rise to HP^--radicals.

It is believed that the destruction of the radical state of TAN is produced by the HP radicals directly, but it cannot be excluded that the reaction is mediated by radicals derived from pyrogallol. However, it should be emphasized that the present method can be used to monitor the yields of HP radicals even if it is the radicals of the reducing agents (pyrogallol) that cause the destruction. The latter radicals are produced with the same yield as HP^- and therefore the rate of degradation of TAN will be proportional to the yield of HP^- also in this case.

The present method is not a diagnostic test for porphyrin anion radicals, since other reducing agents may quench the spins of nitroxide radicals. However, the method, which is reproducible and simple, may be very useful in the elucidation of the photoinduced reactions of porphyrins in biological systems. Firstly, electron transfer from biomolecules to excited prophyrin molecules may be studied. Secondly, reactions of porphyrin free radicals with biomolecules may be conveniently observed.

REFERENCES

1. Grannelli, S. G., Diamond, J., McDonagh, A. F., Wilson, C. B. and Nielsen, S. L. *Cancer Res. 35* (1975) 2567.
2. Dougherty, T. J., Grindey, G. B., Fiel, R., Weishaupt, K. R. and Boyle, D. G. *J. Natl. Cancer Inst. 55* (1975) 115.
3. Kelly, J. F., Snell, M. E. and Berenbaum, M. C. *Br. J. Cancer 31* (1975) 237.
4. Dougherty, T. J., Lawrence, G., Kaufman, J. H., Boyle, D., Weishaupt, K. R. and Goldfarb, A. *J. Natl. Cancer Inst. 62* (1979) 231.
5. Weishaupt, K. R., Gomer, C. J. and Dougherty, T. J. *Cancer Res. 36* (1976) 2326.
6. Moan, J., Pettersen, E. O. and Christensen, T. *Br. J. Cancer 39* (1979) 398.
7. Cannistraro, S. and Van de Vorst, A. *Biochem. Biophys. Res. Commun. 74* (1977) 1177.
8. Rozantsev, E. G., Ed., *Free Nitroxyl Radicals*, Plenum, New York – London 1970, pp. 213 – 214.
9. Cauzzo, G., Gennari, G., Jori, G. and Spikes, J. D. *Photochem. Photobiol. 25* (1977) 389.
10. Eisenstein, K. K. and Wang, J. H. *J. Biol. Chem. 244* (1969) 1720.
11. Dougherty, T. J., Gomer, C. J. and Weishaupt, K. R. *Cancer Res. 36* (1976) 2330.