

## THE APPARENT PRODUCTION OF SUPEROXIDE AND HYDROXYL RADICALS BY HEMATOPORPHYRIN AND LIGHT AS SEEN BY SPIN-TRAPPING

Garry R. BUETTNER and Larry W. OBERLEY<sup>+</sup>

*Wabash College, Crawfordsville, IN 47933 and <sup>+</sup>Radiation Research Laboratory, The University of Iowa, Iowa City, IA 52242, USA*

Received 15 September 1980

### 1. Introduction

Hematoporphyrin is known to have photodynamic properties. The exposure of biomolecules or cells to hematoporphyrin and visible light can result in damage [1] and can even be lethal [2]. This inactivating effect appears to be greater in cancer cells than in corresponding normal cells [3]. This is thought to be due in part to the greater accumulation of hematoporphyrin in malignant tissue than in most other tissues [4–7].

There is evidence that the cytotoxic action of hematoporphyrin and light is the result of the photosensitized production of singlet oxygen, a short-lived, highly reactive state of molecular oxygen [8–10]. However, the interaction of hematoporphyrin with biomolecules may proceed by a radical mechanism rather than by a singlet oxygen mechanism [11]. In [12] evidence indicating the production of hydroxyl radical in cell systems exposed to hematoporphyrin and red light appeared. Hematoporphyrin could produce superoxide radical in aprotic solvents [13], but no free radical formation in the presence of water was observed [13]. The photosensitized production of superoxide by hematoporphyrin has been reported in [14] but only in the presence of NADH or NADPH. Thus, the significance of a radical mechanism for this reaction needs to be thoroughly investigated. In [15–17] we proposed a new model for malignancy. The essence of the model is as follows:

Tumor cells have diminished amounts of the manganese-containing superoxide dismutase, but at the same time they are capable of producing significant amounts of superoxide radical. We believe this difference between malignant and normal cells can be exploited in the treatment of cancer. Agents

which increase the production of superoxide in the cell should be able to inflict lethal damage to the tumor cell because the lowered manganese-superoxide dismutase activity results in a loss of protection from the damaging effects of superoxide.

Here we have used the spin-trapping technique [18] to demonstrate that hematoporphyrin and light produces the superoxide radical in aqueous solution, suggesting that the mode of action of hematoporphyrin may be consistent with the above model.

### 2. Materials and methods

The spin-trapping agent, 5,5-dimethylpyrroline-1-oxide (DMPO) was purchased from Aldrich, Milwaukee, WI. The DMPO was diluted with water and purified as in [19]. The concentration of the stock solution was determined spectrophotometrically ( $\epsilon_{234} = 7700 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) in ethanol.

Superoxide dismutase, catalase and hematoporphyrin dichloride (lot 107c-0356) were obtained from Sigma, St Louis, MO and hematoporphyrin IX (lot 517) was obtained from Porphyrin Products, Logan, UT. These products were used without further purification.

The hematoporphyrin solutions being studied were placed in an ESR spectrometer (Varian E-4) cavity in an aqueous sample cell and then irradiated using a projector with a 500 W 3200 K tungsten bulb.

### 3. Results

The illumination of hematoporphyrin in the presence of 50 mM DMPO resulted in the spectra of fig.1.

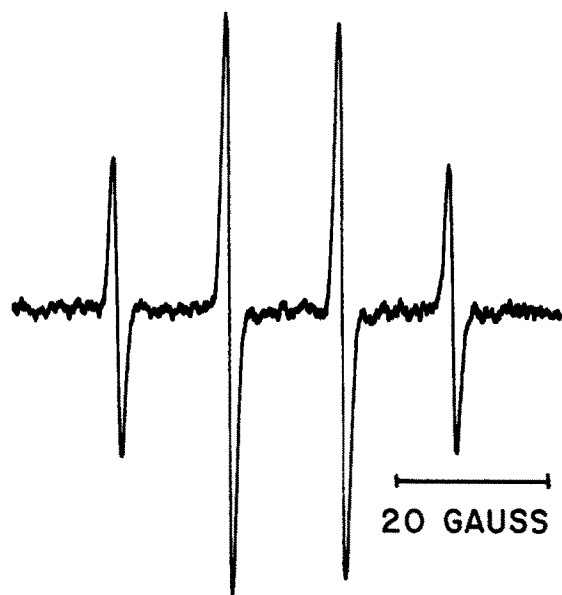


Fig.1. An example of the hydroxyl spin adduct spectra of DMPO observed with hematoporphyrin (either from Sigma or Porphyrin Products) and light. The reaction mixture contained 0.1 mM hematoporphyrin, 50 mM DMPO, 1 mM diethylenetriamine pentaacetic acid in 50 mM phosphate buffer at pH 7.5 ( $a^N = a^H_\beta = 15.0$ ,  $g = 2.0060$ ).

This 1:2:2:1 spectrum ( $g = 2.0060$ ,  $a^N = a^H_\beta = 15.0$  G) is comparable to the spectrum that has been attributed to the hydroxyl radical spin adduct of DMPO [20].

When superoxide dismutase was incorporated into the reaction mixture of fig.1 at 200 units/ml, the ESR signal from the hydroxyl radical spin adduct of DMPO was suppressed. Heat-inactivated superoxide dismutase was without effect. Catalase at 900 units/ml and even at 2500 units/ml produced no significant change in signal intensity from that observed from the solution of fig.1.

When 30% by volume of the water of the solution of fig.1 was replaced with ethyl alcohol, the spectrum of fig.2 results. This spectrum ( $a^N = 15.8$  G,  $a^H_\beta = 22.9$  G and  $g = 2.0067$ ) is identical to the DMPO hydroxyethyl radical spin adduct seen in a microsomal system [21] and in a Fenton system [22]. When superoxide dismutase at 200 units/ml is included in the solution of fig.2, the ESR signal of fig.2 is suppressed.

When 1 M azide is included in the reaction mixture of fig.1, the signal shown in fig.3 is observed. This signal ( $a^{N_1} = 14.9$  G,  $a^H_\beta = 14.9$  G,  $a^{N_2} = 3.0$  G,

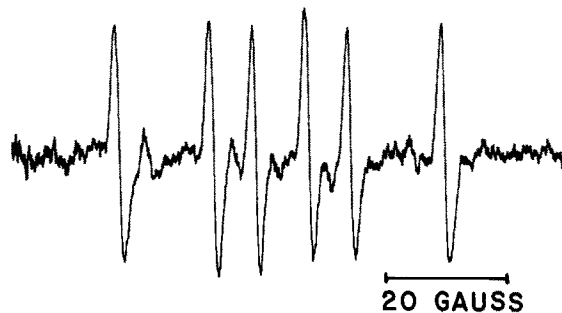


Fig.2. The hydroxyethyl radical spin adduct spectrum of DMPO. The reaction mixture is the same as fig.1 except 30% by vol. of the water in the solution of fig.1 has been replaced by ethanol ( $a^N = 15.8$  G,  $a^H_\beta = 22.9$  G,  $g = 2.0067$ ).

$g = 2.006$ ) with a 1:1:1:2:2:2:2:2:1:1:1 intensity pattern is identical to the signal we have observed in the Fenton system with the inclusion of azide [22]. Using  $^{15}\text{N}$  sodium azide, this signal was identified as the azide radical spin adduct of DMPO [23].

With both ethanol and azide the relative intensities of the spin adduct signals of the hydroxyethyl radical or the azide radical and the hydroxyl radical spin adduct of DMPO vary as the relative concentrations of scavenger and DMPO are varied, i.e., at relatively high scavenger concentration we see only the appropriate scavenger radical spin adduct (fig.2,3) but at relatively low scavenger concentration (compared to

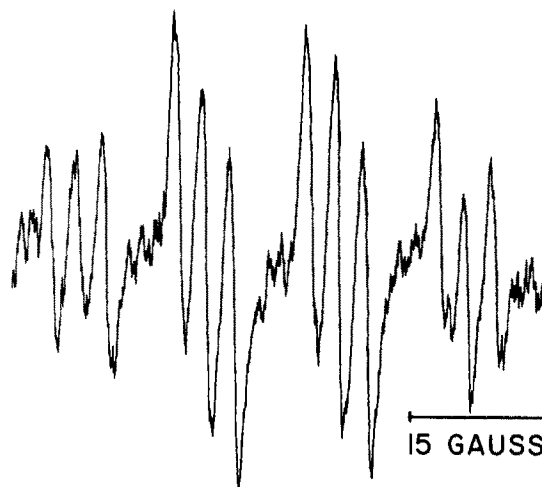


Fig.3. The azide radical spin adduct spectrum of DMPO. The reaction is the same as that of fig.1 except 1 M  $\text{NaN}_3$  has been introduced ( $a^{N_1} = 14.9$  G,  $a^H_\beta = 14.9$  G,  $a^{N_2} = 3.0$  G and  $g = 2.006$ ).

DMPO) both  $\cdot\text{OH}$  and scavenger radical spin adducts can be seen.

The inclusion of 1,4-diazabicyclo [2.2.2] octane (DABCO) at 1 M in the solution of fig.1 decreased the ESR signal to ~50% of the original solution.

Diethylene triamine pentaacetic acid [24] at 1 mM had no effect on the nature of the spin adducts observed in any of the solutions studied; the same spin adducts were observed in the presence or absence of this chelating agent. No signal was observed when hematoporphyrin was excluded from the experimental solution. Similar results were obtained from both the Sigma and Porphyrin Products preparations of hematoporphyrin.

#### 4. Discussion

The observation that superoxide dismutase will suppress the ESR signal observed in the hematoporphyrin-DMPO spin-trapping system indicates that hematoporphyrin can produce the superoxide ion in aqueous solution. However, the hydroxyl spin adduct rather than superoxide spin adduct of DMPO is observed. Two possibilities exist to explain this observation:

- (i)  $\text{O}_2^{\cdot-}$  may act as a precursor for the formation of  $\cdot\text{OH}$ ;
- (ii) The  $\text{O}_2^{\cdot-}$  spin adduct of DMPO is formed but is too short-lived in this system to observe [19]; moreover, to be consistent with our observations, the superoxide spin adduct would have to decay to yield the hydroxyl spin adduct. If this is true, the hydroxyl radical itself is not formed.

It has been shown that the breakdown of the DMPO/OOH spin adduct can result in the formation of the DMPO/OH spin adduct [25] which is relatively long-lived [19]. If the free  $\cdot\text{OH}$  radical is formed, we might expect that in the presence of  $\cdot\text{OH}$  radical scavengers that undergo specific radical-forming reactions to observe the DMPO spin adduct of these new radicals. Indeed, as seen in fig.2 and 3, in the presence of ethanol and azide we see exactly those spin adducts expected if the free hydroxyl radical were present, and as expected the relative intensities of these new spin adducts and the  $\cdot\text{OH}$  spin adduct vary as the relative concentration of DMPO and  $\cdot\text{OH}$  scavengers are varied. These observations are consistent with the hypothesis that the superoxide radical is produced from hematoporphyrin and that the superoxide radicals in turn form hydroxyl radicals.

The inability of catalase to affect the intensity of the signal seen for the solution shown in fig.1 indicates that hydrogen peroxide is not involved in the production of this reactive free radical. Thus the HaberWeiss process [26] appears not to play a part in the production of the reactive hydroxyl-like radical observed. This would indicate the production of hydroxyl radical from superoxide in this system by an unknown mechanism.

That DABCO, a singlet oxygen quencher, reduces the intensity of the DMPO/OH spin adduct is consistent with singlet oxygen being involved in the production of superoxide. However, DABCO could easily be acting as a radical scavenger. For example, triethylamine reacts with  $\cdot\text{OH}$  with a rate constant of  $1.1 \times 10^{10}$  [27]; thus, the role of singlet oxygen in this process cannot yet be resolved.

If our model for malignancy is correct, we should expect those agents that increase the flux of  $\text{O}_2^{\cdot-}$  in the cell to have a greater toxicity in cancer cells than normal cells. Ozone, which can also produce oxygen free radicals, selectively inhibits growth of human cancer cells in comparison to normal cells [28]. Indeed, many of the anticancer drugs appear to generate the superoxide or hydroxyl radicals when activated appropriately [29-32]. Thus, the observation that hematoporphyrin can produce the superoxide radical is consistent with our suggestion that a differential effect should be expected with those agents that increase the production of  $\text{O}_2^{\cdot-}$  in the cell. The facts that tumor cells have lower superoxide dismutase activity than corresponding normal cells and that hematoporphyrin preferentially kills tumor cells over normal cells is consistent with the following two hypotheses:

- (1) Hematoporphyrin exerts its photodynamic effect by a radical mechanism;
- (2) The selective killing of tumor cells over normal cells by hematoporphyrin is caused not only by a greater accumulation in tumor cells over normal cells but also by the lower superoxide dismutase activity in tumor cells.

#### Acknowledgements

This work was supported in part by the Research Corporation, the Haines Fund and by a Grant-in-Aid of Research from Sigma Xi, The Scientific Research Society.

## References

- [1] Fowlks, W. L. (1959) *J. Invest. Dermatol.* 32, 233.
- [2] Sery, T. W. (1979) *Cancer Res.* 39, 96–100.
- [3] Dougherty, T. J., Kaufman, J. E., Goldfarb, A., Weishaupt, K. R., Boyle, D. and Mittleman, A. (1978) *Cancer Res.* 38, 2628–2635.
- [4] Gregorie, H. B., Horger, E. O., Ward, J. L., Green, J. F., Richards, T., Robertson, H. C. and Stevenson, T. B. (1968) *Ann. Surg.* 167, 820–827.
- [5] Rasmussen-Taxdall, D. S., Ward, G. E. and Figge, F. H. (1955) *Cancer* 8, 78–81.
- [6] Winkleman, J. and Rasmussen-Taxdall, D. S. (1960) *Bull. John Hopkins Hosp.* 107, 228–233.
- [7] Dougherty, T. J., Grindey, G. B., Fiel, R., Weishaupt, K. R. and Boyle, D. G. (1975) *J. Natl. Cancer Inst.* 55, 115–119.
- [8] Weishaupt, K. R., Gomer, C. J. and Dougherty, T. J. (1976) *Cancer Res.* 36, 2326–2329.
- [9] Dougherty, T. J., Gomer, C. J. and Weishaupt, K. R. (1976) *Cancer Res.* 36, 2330–2333.
- [10] Moan, J., Pettersen, E. O. and Christensen, T. (1979) *Brit. J. Cancer* 39, 398–407.
- [11] Cannistraro, S. and Van de Vorst, A. (1977) *Biochem. Biophys. Res. Commun.* 74, 1177–1185.
- [12] Hariharan, P. V., Courtney, J. and Eleczko, S. (1980) *Int. J. Radiat. Biol.* 37, 691–694.
- [13] Cox, G. S., Whitten, D. G. and Giannotti, C. (1979) *Chem. Phys. Lett.* 67, 511–515.
- [14] Frenkel, A. W. (1980) *Proc. Int. Conf. Oxygen and Oxy Radicals II*, Austin in press.
- [15] Oberley, L. W. and Buettner, G. R. (1979) *Cancer Res.* 39, 1141–1149.
- [16] Oberley, L. W., Oberley T. D. and Buettner, G. R. (1980) *Med. Hypoth.* 6, 249–268.
- [17] Buettner, G. R. and Oberley, L. W. (1980) *Proc. Int. Conf. Oxygen and Oxy Radicals II*, Austin in press.
- [18] Janzen, E. G. (1971) *Acc. Chem. Res.* 4, 31–40.
- [19] Buettner, G. R. and Oberley, L. W. (1978) *Biochem. Biophys. Res. Commun.* 83, 69–74.
- [20] Harbour, J., Chew, V. and Bolton, J. R. (1974) *Can. J. Chem.* 52, 3549–3553.
- [21] Lai, C. and Piette, L. H. (1977) *Biochem. Biophys. Res. Commun.* 78, 51–59.
- [22] Buettner, G. R. and Oberley, L. W. (1980) unpublished.
- [23] Kremers, W. and Singh, A. (1980) *Can. J. Chem.* 58, in press.
- [24] Buettner, G. R., Oberley, L. W. and Luethauser, S. H. W. C. (1978) *Photochem. Photobiol.* 28, 693–695.
- [25] Finkelstein, E., Rosen, G. M., Rauckman, E. J. and Paxton, J. (1979) *Mol. Pharmacol.* 16, 676–685.
- [26] Haber, F. and Weiss, J. (1934) *Proc. Roy. Soc. Lond. A* 147, 332–351.
- [27] Farhataziz and Ross, A. G. (1977) Selected Specific rates of reactions of transients from water in aqueous solution, US Dept. Commerce/National Bureau of Standards NSRDS-NBS-59.
- [28] Sweet, F., Kao, M. S. and Lee, S. C. D. (1980) *Science* 209, 931–933.
- [29] Bachur, N. R., Gordon, S. L. and Gee, M. V. (1976) *Cancer Res.* 38, 1745–1750.
- [30] Lown, J. W., Sim, S. K. and Chen, H. H. (1978) *Can. J. Biochem* 56, 1042–1047.
- [31] Sausville, E., Peisach, J. and Horwitz, S. (1978) *Biochemistry* 17, 2740–2746.
- [32] Oberley, L. W. and Buettner, G. R. (1979) *FEBS Lett.* 97, 47–49.