EPR Studies of Hypericin. Photogeneration of Free Radicals and Superoxide

Lev Weiner[a] **and Yehuda Mazur**[b]
Departments of [a]Chemical Physics and [b]Organic Chemistry, Weizmann Institute of Science, 76 100 Rehovot, Israel

Hypericin, a potent antiviral agent, displays, in the absence of light and electron donors, an EPR signal which is attributed to a semiquinone-like radical, formed by intermolecular electron transfer. On irradiation with visible light the amplitude of the EPR signal increases significantly. This increase is highest (*ca.* 20 fold) in aqueous dispersions of the lysine salt of hypericin. Irradiation of hypericin water aggregates in the presence of oxygen generates superoxide radicals which may be registered by the spin trap technique.

This finding implies that the free radicals of hypericin and superoxide radicals formed on photoirradiation of hypericin may play a hitherto unrecognized role in the biological activities elicited by hypericin both *in vivo* and *in vitro*.

Hypericin, a naphthodianthrone derivative (Fig. 1), has been found to be a potent antiviral agent.[1] Its span of activity

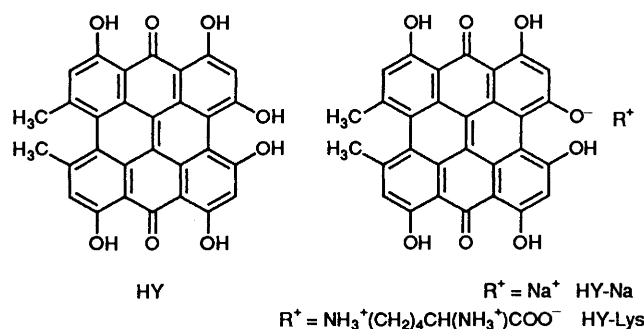


Fig. 1 Structures of hypericin, HY, sodium salt of hypericin (HY-Na) and lysine salt of hypericin (HY-Lis)

encompasses membrane viruses,[2] including HIV.[1] Hypericin is also reported to be a putative antineoplastic agent since it inhibits mitochondrial succinoxidase.[3] These biological activities are to a large extent light dependent. Photosensitizing properties of hypericin, a constituent of plants of the *Hypericum* genus, are also known to cause *in vivo* hypericism, a disease of grazing animals.[4] Extreme sensitivity to solar radiation was found in a ciliate, *Stentor coeruleus* containing hypericin linked to an apoprotein.[4–6]

It has been postulated that singlet oxygen ($^1O_2$) is responsible for most of these photodynamic activities.[4,5] Its involvement in antiretroviral activity of hypericin was shown by the inhibition of its activity in the presence of azide or crocetin.[7] It was observed that $^1O_2$ participates in the hypericin-promoted mitochondrial inhibition of succinoxidase.[3]

Hypericin photosensitization in ethanol and lipid micelle solutions (in the presence of O_2) generates $^1O_2$ which proved to be the main photo product.[8,9] The possibility of superoxide formation was estimated to be very low since the sum of the quantum yields of $^1O_2$ production and of hypericin fluorescence was found to be close to unity.[9]

This photochemical behaviour differs from that of anthraquinones, including antitumour antibiotics, which generate mainly superoxide and hydroxyl radicals in light-stimulated reactions.[10] Oxygen radicals are involved in numerous biological processes such as lipid oxidations, DNA scissions, inhibition of cell proliferation *etc.*[10]

Hypericin (HY) (Fig. 1) is a fluorescent non-ionic acidic compound having a pK of *ca.* 4.5, which is only slightly soluble in most organic solvents, and very slightly soluble in water. At pH 4.5–11 it undergoes ionization and gives red monobasic salts with both inorganic and organic bases, whose longest wavelength maximum is at 590 nm (ethanol).[11] In this form HY is soluble in organic polar solvents like ethanol, acetone, ethyl acetate, DMF *etc.* and disperses in water. The sodium salt of hypericin (HY-Na) (Fig. 1) gives violet non-fluorescent dispersions whose longest wavelength maximum lies at 600 nm, consisting of large high molecular aggregates of *ca.* 50–200 molecules, non-structured, containing water.[11,12] The lysine salt of hypericin (HY-Lys) (Fig. 1) gives different dispersed aggregates which are yellow-brown, also non-fluorescent, but their respective maximum is at 500 nm. The blue shift of *ca.* 100 nm indicates the proximity of the molecules in these HY-Lys aggregates.

In the present work we describe the detection of EPR signals of hypericin in water and in ethanol, which were found to be stable in the presence of air. On irradiation by visible light an increase in the concentration of the free radicals was detected in the absence of electron donors. Finally, using the spin traps technique, we showed that irradiation with visible light of hypericin in water generates superoxide radicals.

Experimental

Hypericin was obtained by synthesis.[12] The water dispersions of hypericin sodium salt (HY-Na) were prepared by sonication of the dry powdered hypericin with 1 mol equiv. of aqueous sodium hydrogencarbonate, followed by filtration through a 0.45 μm millipore filter. The pH of the resulting solution was *ca.* 7.5; the water dispersions of hypericin–lysine salt (HY-Lys) were prepared by sonication of the dry powdered hypericin with *ca.* 1.1 mol equiv. of aqueous solution of lysine followed by centrifugation (g = 50 000), and filtration through a 0.45 μm millipore filter. The pH of the resulting solution was *ca.* 8.5; the concentrations of HY-Na in ethanol were determined from the visible spectra, using as a standard the molar extinction coefficient of 48 000 at λ_{max} 590 nm.[12] At these concentrations HY-Na in ethanol exists as a monomer whose absorbance is directly proportional to its concentration. The concentrations of HY-Na and HY-Lys in water were determined in the same way after ten-fold dilution with ethanol, whereby the water aggregates were converted to monomers, whose absorbance was used as a measure of the compound concentration.

Catalase and superoxide dismutase (SOD) were purchased

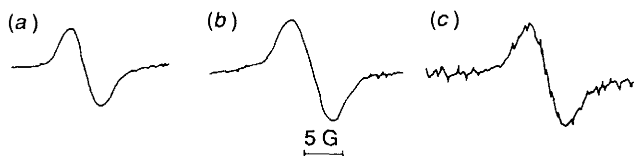


Fig. 2 EPR spectra of hypericin solutions. (*a*) HY-Lys in water, 1.1×10^{-3} mol dm^{-3}; concentration of paramagnetic species (CPS), 4×10^{-6} mol dm^{-3}. (*b*) HY-Na in ethanol, 4×10^{-3} mol dm^{-3}; CPS, 1.8×10^{-6} mol dm^{-3}. (*c*) HY-Na in water, 2×10^{-3} mol dm^{-3}; CPS, 5×10^{-7} mol dm^{-3}. EPR conditions: microwave power, 20 mW; modulation amplitude, 3.2 G; receiver gain: (*a*) 10^3, (*b*) 5×10^3, (*c*) 4×10^4. (The dependence of peak intensity of the EPR signal has shown that under experimental conditions there is no saturation).

Table 1 Stimulation of free radical production in hypericin solutions by visible light[a]

Sample	Solvent	Enhancement[b]
HY-Lys	water	21.5
HY-Lys	ethanol	3.3
HY-Na	water	3
HY-Na	ethanol	2.7

[a] Ambient temperature; [HY] = 2×10^{-3} mol dm^{-3}. [b] EPR signal peak intensity relative to that in the absence of light.

from Sigma (St. Louis, MO). 5,5-Dimethyl-1-pyrroline *N*-oxide, DMPO (Sigma), was purified as described.[13] The degree of DMPO purity was determined by the EPR spectroscopic method.

EPR spectra were recorded using an E-12 spectrometer (Varian) with 100 kHz field modulation operating at 9.5 GHz in a flat quartz cell (v = 200 mm^3). Photoinduced EPR spectra of directly illuminated samples were recorded inside a microwave cavity, in front of which a focussing lens was placed. An HMBO 200 W superpressure mercury lamp was used as a light source equipped with a glass filter (1.5 cm thick) containing 1% aqueous $CuSO_4$. The photodiode CLT-2160088 was calibrated using the radiometer OPHIR, model DG, OPHIR Optics Ltd., Jerusalem, and then introduced directly into the cavity. The visible light power was *ca.* 10 mW cm^{-2}. Samples were deoxygenated by bubbling nitrogen gas for 10 min through sample solutions (v = 0.4 cm^3). Concentration of the radicals was estimated by the double integration method. The radical TEMPO,* at a given concentration, was employed as a reference. The accuracy of determination of radical concentrations was 20%.

Results

Solutions of HY-Na in ethanol, and HY-Na and HY-Lys in water give EPR singlet spectra with a linewidth of ΔH = 3.5–5.2 G and $g = 2.0032 \pm 0.0001$ (Fig. 2). The HY-Na salts show similar integral intensity of their EPR spectra, while for HY-Lys the integral intensity is 5–7 times higher.

On addition of $NaBH_4$ to a solution of HY-Na in ethanol, or of either β-mercaptoethanol, or KO_2 in crown ether (5 mmol dm^{-3}) to a solution of HY-Na in dimethylformamide (DMF), the EPR signal was enhanced by a factor of 2–4. However introduction of reducing agents used in biological systems such as ascorbate, glutathione and cysteine (1 mmol dm^{-3}) to an aqueous dispersion of HY-Na or HY-Lys did not lead to any increase of the signal intensity.

Stimulation of EPR signals was also observed on irradiation of HY with visible light: signals due to HY-Na in ethanol and in water were enhanced by a factor of *ca.* 3. However HY-Lys in water showed a signal whose intensity was increased *ca.* 20 times (Table 1).

* 2,2,6,6-Tetramethylpiperidin-1-yloxy radical.

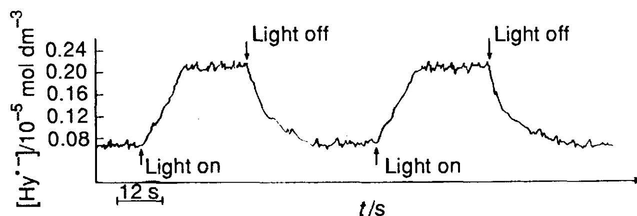


Fig. 3 Kinetics of the photoinduced generation of the EPR spectrum of radical species from HY-Na (2×10^{-3} mol dm^{-3}) in ethanol and its decay in the dark. EPR conditions: microwave power, 20 mW; modulation amplitude, 3.2 G; receiver gain, 4×10^4.

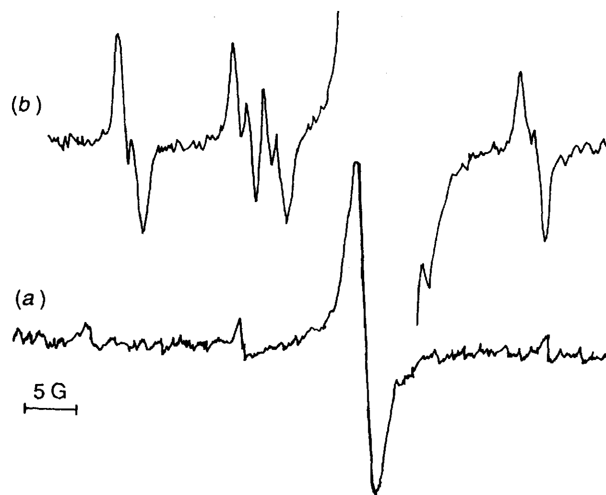


Fig. 4 EPR spectra from aerated water dispersion containing HY-Lys (3.5×10^{-4} mol dm^{-3}) and DMPO (8×10^{-2} mol dm^{-3}). (*a*) Dark, and (*b*) during photoirradiation. EPR conditions: microwave power, 20 mW; modulation amplitude, 0.63 G; time constant, 0.3 s; time scan, 240 s; receiver gain, 10^4.

We have measured the kinetics of the photoinduced generation of radicals of HY-Na in ethanol and HY-Lys in water, and their decay in the dark (Fig. 3). The kinetics reflect a fast increase of radical concentration, a rapid approach to the plateau, and a slow decrease after the light is extinguished. The kinetics of this decay for HY-Na and for HY-Lys are satisfactorily described by the second-order reaction constants, $k_1 = 8 \times 10^4$ dm^3 mol^{-1} s^{-1} and $k_1 = 3.5 \times 10^4$ dm^3 mol^{-1} s^{-1} respectively. However, for the latter this applies only in the early stage of the decay curve.

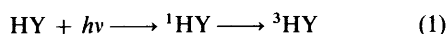
Visible light irradiation of aerated aqueous dispersions of HY-Na and HY-Lys in the presence of a spin trap, DMPO, generates an EPR spectrum of a DMPO/$\dot{O}$OH adduct with a_N = 14.2 G, a_H^β = 11.5 G, a_H^γ = 1.25 G (Fig. 4). The values of the hyperfine splitting constants are characteristic of the above adduct in water.[14] In the presence of SOD (30 μg cm^{-3}) no spin adduct was observed, while catalase (50 units cm^{-3}) exerted very little effect on this adduct.

Discussion

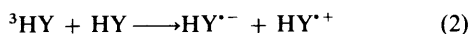
In the present work we show that HY both in ethanol solution and in aqueous dispersions, possesses a radical character. The structure of the radical is as yet not clear to us, although its EPR parameters (*g* factors and linewidth) resemble those of a semiquinone radical.[15] The enhancement of EPR signal amplitude in the presence of reducing agents in ethanol and DMF supports this assignment. However the EPR spectra of HY

which were observed both in air-saturated and in nitrogen-gassed ethanol solutions, or aqueous dispersions, lacked the hyperfine splitting due to interaction of the unpaired electron with HY protons. This may be explained by the fact that HY in water is in the aggregated form, which lowers the mobility of the HY molecules resulting in the broadening of the EPR signal. It is also possible that HY in ethanol, in the concentrations used in the experiments (> 1 mmol dm^{-3}), forms aggregates which also broaden the EPR signals. An additional explanation for the unresolved EPR signal may be that it corresponds to a superposition of several spectra due to a number of semiquinone radicals. It is plausible that the origin of the observed radical species is an intermolecular electron-transfer from the quinone fragment of a HY molecule to the hydroquinone fragment of a neighbouring molecule (Fig. 1), leading either to a cation radical and a semiquinone anion radical (electron migration), or to two semiquinone-type radicals (H atom migration). A comparable spontaneous appearance of radicals was observed in a solution of adriamycin.[16]

The light stimulation of the EPR signals of hypericin (Table 1, Fig. 3) may also be explained by an intermolecular electron transfer mechanism. On irradiation of HY, a triplet state is formed which may transfer its electron to the HY in the ground state to give HY cation and anion radicals. This mechanism was postulated to occur on photoexcitation of concentrated solutions of quinones (≥1 mmol dm^{-3}) in the absence of electron donors[17] [eqns. (1) and (2)].



$$HY + h\nu \longrightarrow {}^1HY \longrightarrow {}^3HY \quad (1)$$



$$^3HY + HY \longrightarrow HY^{\bullet -} + HY^{\bullet +} \quad (2)$$

The second-order kinetics of the disappearance of the photo-induced EPR signals is probably due to the recombination of the formed radicals, to give ground state molecules. However the bimolecular rate constant for this recombination ($k_1 \lesssim 10^5$ dm^3 mol^{-1} s^{-1}) is considerably lower that the known recombination rate constant for the semiquinone of daunomycine (k_1 *ca.* 10^8 dm^3 mol^{-1} s^{-1}),[18] 1,5- and 1,8-dihydroxy-9,10-anthrasemiquinones (k_1 *ca.* 10^9 dm^3 mol^{-1} s^{-1}).[19] The *ca.* 20-fold increase observed on irradiation of aqueous HY-Lys aggregates is most probably due to the short distance between the HY molecules, which are possibly stacked intermolecularly, rendering the electron transfer process more efficient. An alternative explanation for this increase of the intensity of the EPR signal is the participation of a free amino group which may be present in aqueous HY-Lys aggregates. The free amine may transfer an electron to the triplet state of the quinone.[20]

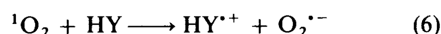
The spin trapping experiments on irradiation of aqueous HY aggregates using DMPO, point to the presence of a superoxide molecule (Fig. 4). The absence of an EPR signal due to the DMPO/ȮOH adduct in the presence of SOD confirms the generation of superoxide molecules.

The formation of superoxide may be rationalized by one of the following processes:

$$(a)\ HY^{\bullet -} + O_2 \longrightarrow HY + O_2^{\bullet -} \quad (3)$$

$$(b)\ {}^3HY + O_2 \longrightarrow HY^{\bullet +} + O_2^{\bullet -} \quad (4)$$

$$(c)\ {}^3HY + O_2 \longrightarrow {}^1O_2 + HY \quad (5)$$



$$^1O_2 + HY \longrightarrow HY^{\bullet +} + O_2^{\bullet -} \quad (6)$$

In process (*a*) the triplet of HY formed on irradiation [eqn. (1)] collides with another HY molecule in the ground state, resulting in an electron transfer. The resulting semiquinone anion radical transfers its electron to oxygen, producing a superoxide [eqn. (3)]. Radical anions derived from anthraquinone or adriamycin are known to react rapidly with oxygen with a rate constant of the order of 10^8 dm^3 mol^{-1} s^{-1}.[19,21] Alternatively, [process (*b*)] the HY triplet may transfer its electron directly to the oxygen molecule, to give superoxide radical. In addition to these Type I mechanisms, a Type II mechanism [process (*c*)] may take place. This process involves a primary generation of singlet oxygen [eqn. (5)] which in turn may oxidize a ground state HY molecule [eqn. (6)]. The generation of $^1O_2$ by hypericin in mitochrondria has been reported recently.[3]

Since we have found that the concentrations of the DMPO/ȮOH spin adducts formed under irradiation of HY-Lys or HY-Na water dispersions are similar, whereas the concentration of the radical anion of HY-Lys is higher by a factor of ten (Table 1), process (*b*) [eqn. (4)] seems to be the preferred one. Although there is no data on $^1O_2$ generation on photoirradiation of hypericin in aqueous dispersions, the possibility that $^1O_2$ participates in the formation of superoxide radicals [process (*c*), eqns. (5) and (6)] cannot be excluded.

Conclusions

Our studies have shown that monobasic salts of hypericin, which are soluble in ethanol and disperse in water, forming aggregates, display an EPR signal in the absence of light. This signal is attributed to a semiquinone-like radical. On irradiation with visible light the intensity of the EPR signal increases. Upon irradiation of aqueous dispersions of HY in the absence of reducing agents, generation of $O_2^{\bullet -}$ radicals was observed by the spin trap technique.

It has been previously shown that singlet oxygen is at least partly responsible for the biological activities elicited by HY. Our experiments indicate that free radicals of hypericin and oxygen radicals generated by photoirradiation of hypericin may also be implicated in the mechanism of these activities.

Acknowledgements

Generous financial support for this work was provided by ViMRx Pharmaceuticals Inc., Stamford, CT, USA and by Israeli Ministry of Absorption (L. W.).

References

1 (*a*) D. Meruelo, G. Lavie and D. Lavie, *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 5230; (*b*) G. Lavie, F. Valentine, B. Levin, Y. Mazur, G. Gallo, D. Lavie, D. Weiner and D. Meruelo, *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 5963.
2 (*a*) G. A. Kraus, D. Pratt, J. Tossberg and S. Carpenter, *Biochem. Biophys. Res. Commun.*, 1990, **172**, 143; (*b*) J. Tang, J. M. Colacino, S. H. Larsen and W. Spitzer, *Antivir. Res.*, 1990, **13**, 313; (*c*) R. F. Sehinazi, Ch. K. Chu, J. R. Babu, B. J. Oswald, V. Saalman, D. L. Cannon, B. F. Eriksson and N. Nasr, *Antivir. Res.*, 1990, **13**, 265; (*d*) I. Lopez-Bazzocchi, J. B. Hudson and G. H. N. Towers, *Photochem. Photobiol.*, 1991, **54**, 95; (*e*) S. Carpenter and G. A. Kraus, *Photochem. Photobiol.*, 1991, **53**, 169.
3 C. Thomas, R. S. MacGill, G. C. Miller and R. S. Pardini, *Photochem. Photobiol.*, 1992, **55**, 47.
4 (*a*) A. C. Giese, *Photochem. Photobiol. Rev.*, 1980, **5**, 229; (*b*) J. P. Knox and A. D. Dodge, *Plant Cell Environ.*, 1985, **8**, 19; (*c*) N. Duran and P. S. Song, *Photochem. Photobiol.*, 1986, **43**, 677.
5 K. C. Yang, R. K. Prusti, E. B. Walker, P. S. Song, M. Watanabe and M. Furuya, *Photochem. Photobiol.*, 1986, **43**, 305.
6 D. P. Häder and M. A. Häder, *Photochem. Photobiol.*, 1991, **54**, 423.
7 D. Meruelo, S. Degar, B. Levin, D. Lavie, Y. Mazur and G. Lavie, 1991, abstract 1-291, HIV Disease: Pathogenesis and Therapy, University of Miami.
8 P. Jardon, N. Lazortchak and R. Gautron, *J. Chim. Phys. Phys.-Chim. Biol.*, 1987, **84**, 1141.
9 H. Racinet, P. Jardon and R. Gautron, *J. Chim. Phys. Phys.-Chim. Biol.*, 1988, **85**, 971.

10 G. Powis, *Free Radicals Biol. Med.*, 1989, **6**, 63 and references cited therein.
11 L. Liebes, Y. Mazur, D. Freeman, D. Lavie, G. Lavie, N. Kudeer, S. Mendoza, B. Levin, B. H. Hochster and D. Meruelo, *Anal. Biochem.*, 1990, **195**, 77.
12 D. Lavie, D. Freeman, H. Bock, J. Fleischer, K. van Kranburg, Y. Ittah, Y. Mazur, G. Lavie, L. Liebes and D. Meruelo, IUPAC, Trends in Medicinal Chem. 90, Proceedings X International Symposium on Medicinal Chemistry, 1990, p. 321.
13 G. R. Buettner and L. W. Oberley, *Biochem. Biophys. Res. Commun.*, 1978, **83**, 69.
14 J. R. Harbour and M. L. Hair, *J. Phys. Chem.*, 1978, **82**, 1397.
15 J. A. Pedersen, *Handbook of EPR Spectra from Quinones and Quinols*, CRC Press, Boca Raton, 1985.
16 D. D. Pietronigro, J. E. McGinnes, M. J. Koren, R. Crippa, M. L. Seligman and H. B. Demopoulos, *Physiol. Chem. Physics*, 1979, **11**, 405.
17 J. H. Bruce, in *The Chemistry of Quinoid Compounds*, ed. S. Patai, Wiley, New York, 1974, vol. I, p. 465.
18 C. Houee-Levin, M. Gardes-Albert, C. Ferradini, M. Farragi and M. Klapper, *FEBS Lett.*, 1985, **179**, 46.
19 H. Pal, D. K. Palit, T. Mukheryee and J. P. Mittal, *J. Chem. Soc., Faraday Trans.*, 1991, **87**, 1109.
20 S. G. Cohen, A. Parola and G. H. Parsons, *Chem. Rev.*, 1973, **73**, 141.
21 J. Butler, B. M. Moley and A. J. Swallow, *FEBS Lett.*, 1985, **182**, 95.

Paper 2/01825J
Received 30*th March* 1992
Accepted 29*th May* 1992