Photoinduced Formation of Reactive Oxygen Species from the Acid Form of 6-(Hydroxymethyl)pterin in Aqueous Solution

by **Andrés H. Thomas**[a]), **Romina Cabrerizo**[a]), **Mariana Vignoni**[a]), **Rosa Erra-Balsells**[b]), **Franco M. Cabrerizo***[b]), and **Alberto L. Capparelli***[a])

[a]) Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CONICET, Casilla de Correo 16, Sucursal 4, 1900-La Plata, Argentina (e-mail: alcappa@dalton.quimica.unlp.edu.ar)

[b]) CHIDECAR – CONICET, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, 3p, Ciudad Universitaria, 1428-Buenos Aires, Argentina (e-mail: fcabrerizo@qo.fcen.uba.ar)

The photochemistry of 6-(hydroxymethyl)pterin (HPT; **1**) in aqueous solution (pH 5–6) was investigated by irradiation at 350 nm at room temperature. The photochemical reactions of the acidic form **1a** were followed by UV/VIS spectrophotometry, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and enzymatic methods for the determination of the superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). When **1a** is exposed to UV-A radiation, the intermediates **4** and **4′** are formed reacting with O_2 to yield 6-formylpterin (FPT; **5**) and 6-carboxypterin (CPT; **6**) under formation of $O_2^{\bullet-}$ and H_2O_2 (*Scheme 3*). The quantum yields of the disappearance of HPT (**1a**) and of the formation of the photoproducts **5** and **6** were determined. HPT was investigated for its efficiency in singlet-oxygen ($^1O_2$) production in acidic aqueous solution. The corresponding quantum yield of $^1O_2$ production (Φ_Δ) was 0.15 ± 0.02, as measured by the $^1O_2$ luminescence in the near-IR (1270 nm) upon continuous excitation of the sensitizer. However, $^1O_2$ does not participate in the actual photooxidation of HPT (**1a**) to FPT (**5**) and CPT (**6**).

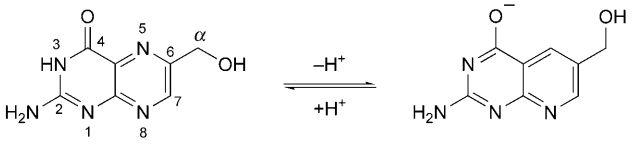
Introduction. – Pterins, *i.e.*, 2-amino-4-hydroxypteridine derivatives, occur in a wide range of biological systems [1][2]. Although pterins are present in only minute amounts in living systems, they are known to participate in important biological functions [1–3] including various photobiological processes such as photoreception in plants [4][5]. For example, the folic acid derivative '5,10-methenyltetrahydrofolate' is present as the light-harvesting antenna in DNA photolyases [6][7], which, in turn, are involved in DNA repair after UV irradiation. Pterins are also interesting as photosensitizers in photochemical processes taking place in organisms exposed to UV-A radiation, and are known to participate as sensitizers in photochemical reactions that induce DNA damage [8–10].

Many pterin derivatives are good singlet-oxygen ($^1O_2$) sensitizers in aqueous solutions [11–13]. Singlet oxygen, the lowest electronic excited state of molecular oxygen, is an important reactive oxygen species (ROS) and one of the main species responsible for the damaging effects of light on biological systems (photodynamic effects) [14][15]. The $^1O_2$ excited state is much more reactive than the triplet ground state ($^3O_2$). Thereby, photosensitization is primarily responsible for the production of $^1O_2$ *in vivo* [16].

Biomedical studies have suggested that pterins are involved in vitiligo, a depigmentation disorder [17][18]. Moreover, different pterin derivatives such as 6- and 7-biopterin are accumulated in the epidermis of affected patients, which leads to a typical fluorescence under *Wood*'s light (351 nm) [17]. Hydrogen peroxide (H_2O_2), another ROS, participates in the pathogenesis of vitiligo, and is also accumulated in the epidermis of affected patients [19].

Pterin proper and its 6-substituted congeners, the most-common derivatives found in nature, behave as weak acids in aqueous solutions. As reported by *Albert* [20], for several pterin derivatives, the dominant equilibrium at pH > 5 involves an amide (acid form) and a phenolate (basic form), as shown in *Scheme 1* for the acid/base equilibrium of 6-(hydroxymethyl)pterin (HPT; **1**). The pK_a of the acid form **1a** and of similarly substituted analogs is *ca.* 8. Note that, in aqueous solution, the protonated 2-amino group or the ring N-atoms have pK_a values below 2 [20].

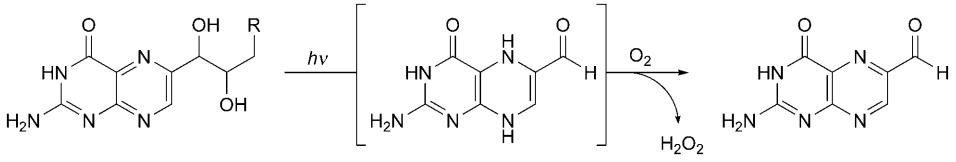
Scheme 1



1a (acid form) **1b** (basic form)

The mechanism involved in the photooxidation of 6-substituted pterins strongly depends on the nature of the substituent at C(6), as well as on the pH. In other words, the photochemistry of the acid and base forms of pterin derivatives are different. Some pterins exhibit a very characteristic photochemistry. For instance, when solutions of biopterin (**2**) and neopterin (**3**), previously purged with Ar gas, are exposed to UV-A radiation, red-colored compounds are generated (*Scheme 2*) [21][22]. It has been proposed that these compounds, with low-intensity long-wavelength absorption bands at *ca.* 480 nm, correspond to 6-acyl or 6-formyl-5,8-dihydropterins such as **4** [21][22]. This would imply reduction of the pterin moiety and oxidation of the substituent *via* an intramolecular redox reaction. Compounds of type **4** are then easily oxidized upon exposure to air or O_2 to yield the corresponding oxidized pterins, *e.g.*, 6-formylpterin (FPT; **5**), together with H_2O_2 (*Scheme 2*).

Scheme 2. *Photooxidation of Biopterin and Neopterin under UV-A Radiation in Aqueous Solution* [21][22]



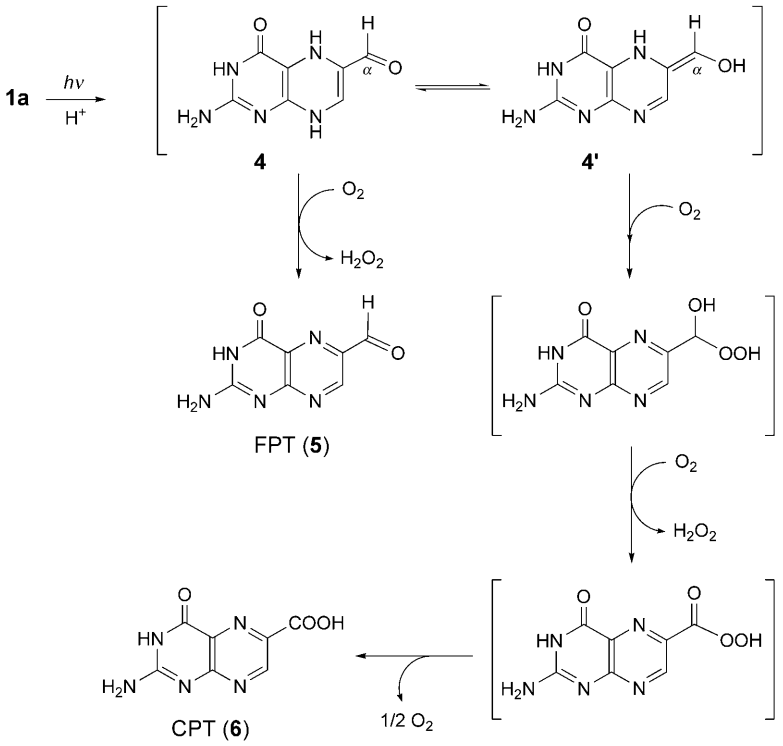
2 R = H (Biopterin)
3 R = OH (Neopterin)

6-Formyl-5,8-dihydropterin

We have recently reported [23] that the photochemical behavior of the basic form **1b** of HPT in aqueous solution is similar to that shown in *Scheme 2*. The photooxidation of HPT to FPT (**5**) occurs *via* at least two steps. In the first step, a photon is absorbed in the absence of O_2, which generates a red-colored intermediate, probably 6-formyl-5,8-dihydropterin. In the second step, this colored intermediate reacts with molecular O_2 to yield FPT and H_2O_2.

As part of our studies of the photochemistry of pterins, we herein report in detail the photochemistry of the acid form **1a** of HPT under UV-A irradiation (350 nm). The results are summarized in *Scheme 3*. We have identified the resulting photoproducts and determined the corresponding quantum yields. The role of molecular O_2 on the mechanisms of these photochemical reactions was analyzed, and the capability of HPT to yield ROS is discussed. In particular, the production of $^1O_2$, $O_2^{\bullet-}$, and H_2O_2 during the photooxidation of HPT was studied, and the results compared with those described earlier for the basic form **1b** and similar 6-substituted pterins.

Scheme 3. *Proposed Reaction Mechanism for the Photooxidation of 6-(Hydroxymethyl)pterin* (**1a**) *in Acidic Aqueous Solution*



Experimental. – *General.* 6-(Hydroxymethyl)pterin (HPT; **1**), 6-formylpterin (FPT; **5**), 6-carboxypterin (CPT; **6**), and other pterins were purchased from *Schircks Laboratories* (Switzerland), and used without further purification. Cytochrome *c* (Cyt) from horse heart, and superoxide dismutase (SOD) from bovine erythrocytes were obtained from *Sigma Chemical Co.* The pH of aq. solns. was adjusted

by adding drops of 0.1–0.2M aq. NaOH or HCl solns. with a micropipette. The ionic strength was *ca.* 10^{-3} M in all experiments. In experiments with singlet oxygen ($^1O_2$), D_2O (>99.9%; *Euriso-top*), DCl (99.5%; *Aldrich*) in D_2O, and NaOD (*CEA*) in D_2O were employed.

Photolysis. Continuous photolysis of HPT (**1**) solns. was performed at pH 5.5 in 1-cm quartz cells at r.t. with a *Rayonet RPR* lamp emitting at 350 nm (*Southern N.E. Ultraviolet Co.*). The experiments were performed in the presence and absence of air. O_2-Free solns. were obtained by bubbling with Ar gas for 20 min.

UV/VIS Analysis. Electronic spectra were recorded on a *Varian Cary-3* spectrophotometer. Measurements were made in quartz cells of 1 cm optical-path length and double cells of 1 cm (for irradiation) and 0.2 cm (for absorbance) length, resp. The absorption spectra of the solns. were recorded at regular intervals of irradiation time, and the signals were averaged and smoothed with the *Varian* software. Experimental-difference (ED) spectra were obtained by subtracting the spectrum at time $t=0$ from the subsequent spectra recorded at different times t. Each ED spectrum was normalized rel. to the maximum abs. value of the absorbance difference, yielding the normalized experimental-difference (NED) spectrum. Reference-difference (RD) spectra and normalized-reference-difference (NRD) spectra were obtained from aq. solns. of commercial standards. The comparison between NED and NRD spectra allows the characterization of the major photolysis products [24][25].

Thin-Layer Chromatography (TLC). TLC Experiments were performed on *DEAE* cellulose plates (100 μm) eluting with 0.3% aq. NH_4Cl soln. Irradiated HPT (**1**) solns. (1.5×10^{-4} M) and aq. solns. of pterin-based standards were developed by exposure to UV/VIS radiation (350 nm), which gave rise to blue fluorescent spots. The corresponding R_f values obtained for pterin proper and several pterin derivatives have been reported before [24].

High-Performance Liquid Chromatography (HPLC). A *System Gold* HPLC setup (*Beckman Instruments*) was used to monitor and quantify the photolysis reaction and photoproducts. A *Pinnacle-II* C_{18} *column* (250×4.6 mm, 5 μm; *Restek*) was used for product separation, eluting with aq. 20 mM potassium phosphate buffer (pH 5.5) containing 2.5 mM EDTA. HPLC Runs were monitored by UV/VIS at 340 nm.

Determination of Quantum Yields. The quantum yields of disappearing HPT (**1a**) and of formed FPT (**5**) and CPT (**6**) were determined in experiments performed under different conditions. *Aberchrome 540* (*Aberchromics Ltd.*) was used as actinometer for the measurements of the incident photon flux P_0 at the excitation wavelength (350 nm). The method for the determination of P_0 has been described in detail elsewhere [26]. Values of the photon flux absorbed by **1a**, *i.e.*, P_a, were calculated from P_0 according to the *Lambert–Beer* law:

$$P_a = P_0\,(1-10^{-A}) \tag{1}$$

where A is the absorbance of **1a** at the excitation wavelength. The evolution of the concentrations of reactant and photoproducts during irradiation was followed by HPLC. Aq. solns. of commercial standards were employed for obtaining the corresponding calibration curves.

Determination of the Concentration of O_2 and H_2O_2. The O_2 consumption during photolysis was measured with an O_2-selective electrode (*Orion 37-08-99*). The exper. setup for these measurements was described elsewhere [27]. For the determination of H_2O_2, a *Cholesterol Kit* (*Wiener Laboratorios S.A.I.C.*) was used. H_2O_2 was quantified after reaction with 4-aminophenazone and phenol [28][29]. Briefly, 400 μl of irradiated HPT (**1a**) soln. was added to 2 ml of reagent. The absorbance at 505 nm of the resulting mixture was measured after 30 min at r.t., using the reagent as blank. Aq. H_2O_2 solns. prepared from commercial standards were employed for obtaining the corresponding calibration curves.

Superoxide-Anion Assay. Photogenerated superoxide anion radicals ($O_2^{\bullet-}$) were detected with the aid of the SOD-inhibitable Cyt reduction [30]. Air-equilibrated HTP (**1a**) solns. were irradiated in the presence of 14 μM Cyt. The extent of Cyt reduction was determined by monitoring the increase in the UV/VIS absorbance at 550 nm. The absorption spectra of oxidized and reduced Cyt are very characteristic, and this spectral change is a sensitive assay for electron-transfer reactions. This assay has been used to detect not only $O_2^{\bullet-}$ in aerated soln., but also electron transfer from excited sensitizers under anaerobic conditions [31].

Determination of Quantum Yields of $^1O_2$ Production. The quantum yield of $^1O_2$ production (Φ_Δ) was determined by direct analysis of the $^1O_2$ near-IR luminescence at 1270 nm [32–34] occurring during continuous irradiation ($\lambda_{ex}=367$ nm) of the aq. soln. of HPT (**1a**). The main features of the method and the equipment have been described in detail before [35–37]. Briefly, a sample soln. in a quartz cuvette is irradiated with a Xe/Hg arc through a H_2O filter, focusing optics, and a monochromator. The $^1O_2$ luminescence is collected with a mirror, chopped, and, after passing through a focusing lens, a cut-off filter (1000 nm), and an interference filter (1271 nm), detected at 90° (with respect to the incident beam) using a cooled NIR photomultiplier (*Hamamatsu*). For determining Φ_Δ, the luminescence signals S_e of solns. of a sensitizer (S_e^S) and a reference sensitizer (S_e^R) were measured. The ratio S_e^S/S_e^R is given by the following equation:

$$\frac{S_e^S}{S_e^R}=\frac{P_0^S}{P_0^R}\frac{\Phi_\Delta}{\Phi_\Delta^R}\frac{k_d}{k_d+k_t^S\,[\text{Sens}]}=\frac{P_0^S}{P_0^R}\frac{\Phi_\Delta^{app}}{\Phi_\Delta^R} \tag{2}$$

where P_0^S/P_0^R is the ratio of the incident photon flux at the excitation wavelength of the sensitizer (S) investigated, and of the reference (R). When k_d, the non-radiative deactivation rate constant, and k_t^S, the rate constant of $^1O_2$ total (physical and chemical) quenching by the sensitizer itself, are known for a given solvent, Φ_Δ may be calculated from *Eqn. 2* by measuring the luminescence signals of the sensitizer and the reference solns., as well as the corresponding incident photon flux. Further, when k_t^S is unknown, an apparent quantum yield of $^1O_2$ production (Φ_Δ^{app}), not corrected for potential $^1O_2$ quenching by the sensitizer itself, is obtained. Because of the short lifetime of $^1O_2$ in H_2O ($\tau_\Delta=1/k_d=3.8$ μs), D_2O was used as the solvent in all experiments ($\tau_\Delta=62$ μs) [38][39]. Measurements with HPT (**1a**) ($\varepsilon_{345}=6.3\times10^3$ M^{-1} cm^{-1}) were carried out at an excitation wavelength of 367 nm in D_2O, where the reference sensitizer phenalen-1(1*H*)-one has a Φ_Δ^R value of 0.97 [37][40].

Continuous Photolysis in H_2O vs. *D_2O.* Solns. of HPT (**1a**) were prepared at the same concentration in both H_2O and D_2O, and irradiated under identical conditions. Solvent effects were then evaluated by comparing the results of the UV/VIS spectrophotometric analyses.

Calculation of Molecular Properties. The ground-state geometry of the acidic form of HPT (**1a**), 6-formyl-5,8-dihydropterin (**4**) and its enol tautomer **4′** (see *Scheme 3*) were calculated with the semiempirical PM3 method, as implemented in HyperChem, vers. 7.01, for Windows [41]. The following parameters were used: restricted *Hartree–Fock* (*RHF*) formalism; total charge, 0; spin multiplicity, 1; convergence limit, 1e-008; iteration limit, 50; accelerate convergence, on; CI method, none; *Polak–Ribiere* algorithm; RMS gradient, 0.006 kcal Å^{-1} mol^{-1}; *in vacuo*. PM3 has proven to be more-effective in studies on molecules containing hetero atoms compared to other methods such as MINDO/3 or MNDO. Heats of formation (ΔH_f), charge densities, and HOMO and LUMO energies were also calculated with the PM3 method (after transferring the optimized geometries): restricted *RHF* formalism; total charge, 0; spin multiplicity, 1; convergence limit, 1e-008; iteration limit, 50; accelerate convergence, on; CI method, single excited; orbital criterion: 6:6. The UV/VIS transition $^1S_0\rightarrow{}^1S_1$ (λ_{max}), and the corresponding oscillator strength (*f*) were calculated after geometry optimization (PM3) with the ZINDO/S (CI, 6:6) method.

Results and Discussion. – 1. *Photolysis in the Presence of O_2.* The spectrum of air-equilibrated solutions of the acidic form of HPT (**1a**) changed significantly with irradiation time, as shown in *Fig. 1,a*. An isosbestic point at 279 nm was observed for solutions irradiated for at least 40 min. No further changes were detected for irradiated solutions stored in the dark. NRD Spectra (see *Experimental*) were obtained by subtracting the spectrum of a standard solution of **1a** from those of standard solutions containing FPT (**5**) and CPT (**6**) in different ratios. As shown in *Fig. 1,b*, the NED spectra were similar to the NRD spectra obtained for a solution containing 15% of **5** and 85% of **6**. This suggests that, under these experimental conditions, CPT (**6**) is the main photoproduct, FPT (**5**) being the side product.

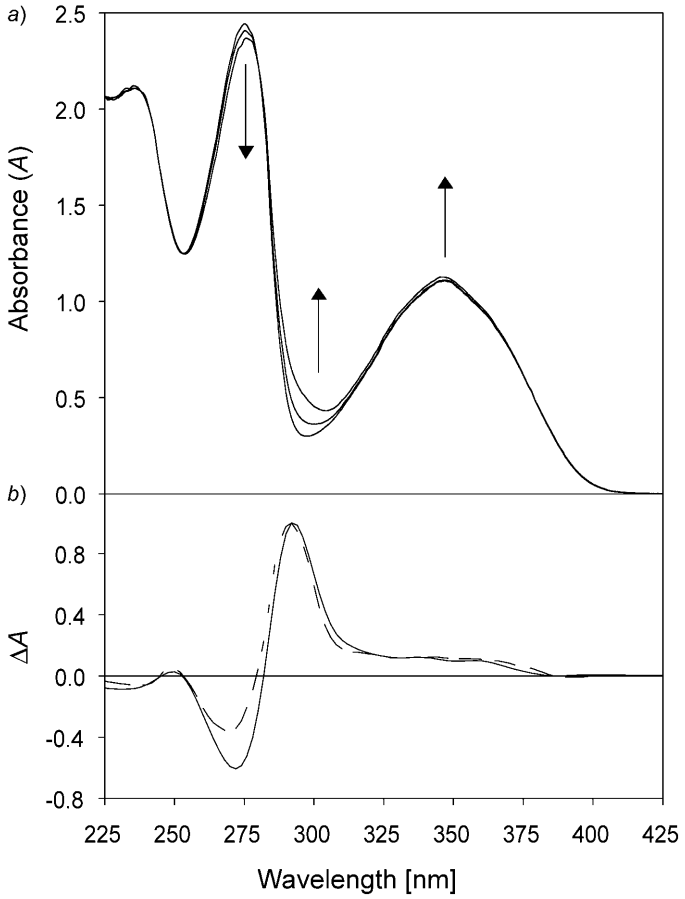


Fig. 1. *UV/VIS Spectroscopic behavior of* **1a** *upon irradiation*. *a*) Evolution of the absorption spectra of irradiated aqueous, aerobic solutions of **1a** (180 μM; pH 5.5) as a function of irradiation time. The three spectra were recorded in intervals of 20 min; arrows indicate the direction of the observed changes at different wavelengths. *b*) NED Spectrum (—) obtained by subtracting the initial UV/VIS spectrum of **1a** from the corresponding spectrum recorded after 20 min of photolysis, and NRD spectrum (- - -) obtained from standard solutions of **1a**, **5**, and **6**. For details, see *Experimental*.

TLC Analysis showed the presence of three fluorescent substances in irradiated HPT (**1a**) solutions at $t=0$, 10, 30, and 55 min. The R_f values were in agreement with those for HPT, FPT and CPT. The presence of FPT and CPT in irradiated solutions of HPT was further confirmed by HPLC experiments. The concentrations of these compounds were determined as a function of irradiation time t. Experiments performed at pH 5.5 with a solution of **1a** at an initial concentration of 200 μM showed that, within the first 15 min of irradiation, the evolution of the concentrations of HPT, FPT and CPT followed a zero-order rate law (*Fig. 2*). Under these conditions, the initial flux of photons absorbed by **1a** (P_a) was $(8.2 \pm 0.4) \times 10^{-4}$ einstein l^{-1} min^{-1}, which corresponds to *ca*. 95% of the incident UV-A radiation. The rate of HPT (**1a**) disappearance was

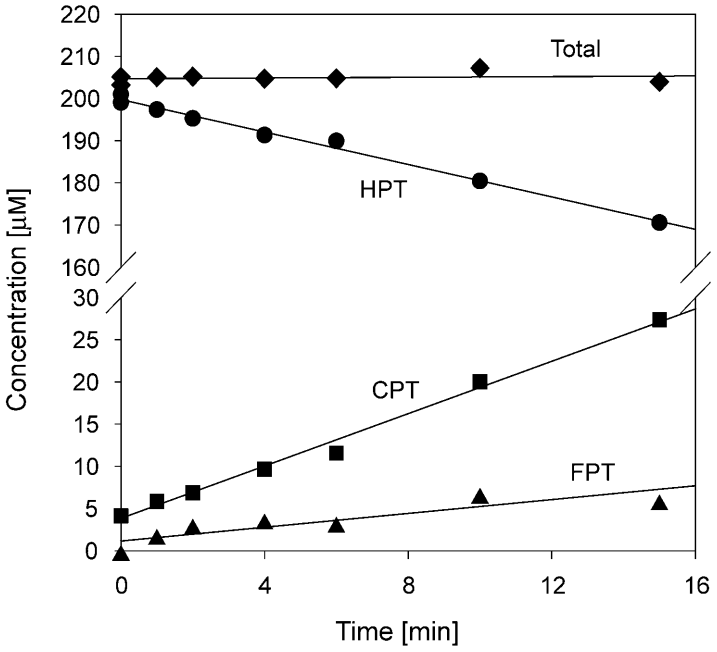


Fig. 2. *HPLC-Based change in the solution concentrations of* **1a** (●), **5** (▲), *and* **6** (■) *upon irradiation of* **1a** (200 μM) *at pH 5.5 under aerobic conditions as a function of irradiation time.* The mathematical sum of the different experimental concentrations (◆) is also shown.

1.9 ± 0.1 μM min^{-1}, and the rates of FPT (**5**) and CPT (**6**) formation were 0.4 ± 0.1 and 1.5 ± 0.1 μM min^{-1}, respectively, the sum of the latter two values being equal to the rate of HPT disappearance. This indicates that FPT and CPT were the main photoproducts, with no other substances being formed in significant amounts. The rates of formation of **5** and **6** were 21 and 79%, respectively, of the rate of the disappearance if **1a**. Therefore, this result was in agreement with the UV/VIS analysis (see above). The quantum yield associated with the disappearance of HPT (**1a**) was calculated as $(2.3 \pm 0.2) \times 10^{-3}$; the values for FPT and CPT formation were $(0.5 \pm 0.1) \times 10^{-3}$ and $(1.8 \pm 0.3) \times 10^{-3}$, respectively.

The above results are surprising, because the only product in the photooxidation of the *basic* form **1b** of HPT is FPT (**5**) [23]. In contrast, the photooxidation of the acid form **1a** yields both FPT and CPT (**6**) (see *Scheme 3* above). At a first glance, this may lead to the conclusion that CPT is formed by photooxidation of FPT. However, we were able to discard this hypothesis because a high percentage of CPT was observed already during the first few minutes of irradiation, when the absorption of HPT at 350 nm is so high that only a small proportion of the incident radiation can be absorbed by FPT. Moreover, experiments performed at higher initial concentrations of HPT showed similar results.

The evolution of O_2 concentration during irradiation was monitored with an O_2 electrode in a closed cell (see *Experimental*). A significant decrease in [O_2] with irradi-

ation time was observed. This result confirms that O_2 present in solution was consumed during photolysis.

H_2O_2 was detected in irradiated solutions of HPT (**1a**), indicating that photooxidation generates this ROS. Its formation was monitored as a function of irradiation time of an aqueous (pH 5.5) 150 μM soln. of **1a** (*Fig. 3*). The estimated quantum yield of H_2O_2 formation was $(2.2 \pm 0.2) \times 10^{-3}$. Comparison of the quantum yield of H_2O_2 formation and that of HPT consumption suggested that, for each molecule of HPT consumed, one molecule of H_2O_2 is generated.

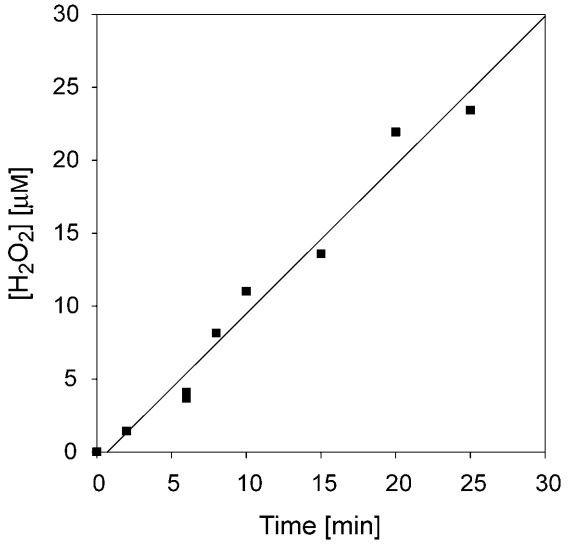


Fig. 3. *Evolution of the H_2O_2 concentration in an irradiated solution of* **1a** (150 μM) *at pH 5.5 as a function of irradiation time*

The generation of H_2O_2 during the photooxidation of other pterin derivatives (biopterin, sepiapterin, FPT, 6-methylpterin, pterin) was previously reported [13][18][42]. The basic form of HPT (**1a**) also produces H_2O_2 under UV-A irradiation [43] in the presence of O_2. Generation of H_2O_2 during the photolysis of pterins is important from a biomedical point of view, and is particularly relevant for skin diseases [19].

2. *Photolysis in the Absence of O_2.* Anaerobic photolysis of **1a** led to the formation of at least one compound with a long-wavelength band and a low molar-absorption coefficient at 480 nm. As soon as air (O_2) was let into the cell, a thermal reaction in the dark was observed, and the mentioned UV/VIS absorption band disappeared. Taking into account the studies carried out by *Pfleiderer* and co-workers on the photochemistry of biopterin and neopterin [21][22][44], and considering the spectroscopic features of related compounds like 5,8-dihydrolumazines [45] and 1,4-dihydropyrazines [46], our results suggest that, as in the case of anaerobic irradiation of the basic form **1b** of HPT [23], an intermediate such as 6-formyl-5,8-dihydropterin is formed. However, the spectroscopic changes at wavelengths above 420 nm were lower than those

observed during the irradiation of **1b**. Therefore, the amount of the intermediate accumulated in the irradiated acidic solutions seems to be very low.

Fast spectroscopic changes were observed when solutions of **1a**, irradiated in the absence of O_2, were aerated. The NED spectrum, obtained by subtracting the spectrum of a non-irradiated solution of **1a** from that of an irradiated and then immediately aerated solution, matched with the NRD spectrum obtained in the same way as that obtained for the photolysis in the presence of O_2 (*e.g.*, for a solution containing 15% of FPT and 85% of CPT). This observation suggests that, under these conditions, FPT and CPT are final, stable compounds generated in the same proportion as under aerobic conditions.

TLC Analysis of the irradiated HPT (**1a**) solutions under anaerobic conditions, followed by aeration, showed three fluorescent spots, with R_f values identical to those for HPT, FPT and CPT. The intensity of the spot of HPT decreased with irradiation time. No other product could be detected by this procedure.

The results obtained by HPLC analysis agreed with those obtained by TLC and UV/VIS analyses. FPT (**5**) and CPT (**6**) were the only photoproducts found in the absence of O_2, followed by immediate aeration (*Fig. 4*). During the first 60 min of irradiation, the change in concentration of HPT, FPT and CPT followed a zero-order rate law (*Fig. 4*). Under these conditions, P_a was determined as $(1.1 \pm 0.1) \times 10^{-4}$ einstein l^{-1} min^{-1}). The quantum yield associated with the disappearance of HPT (**1a**) was $(2.0 \pm 0.3) \times 10^{-3}$, and those due to the formation of FPT (**5**) and CPT (**6**) were $(0.2 \pm 1) \times 10^{-3}$ and $(1.8 \pm 0.3) \times 10^{-3}$, respectively. These values are quite similar to the ones obtained in experiments performed under aerobic conditions, which suggests that the underlying mechanisms are the same.

The determination of H_2O_2 in solutions of 180 μM HPT at pH 5.5, immediately aerated after different times of irradiation under *anaerobic* conditions, revealed that this ROS is also generated. The obtained value for the quantum yield of H_2O_2 production was $(2.5 \pm 0.2) \times 10^{-3}$ for the first 50 min of irradiation. This value is similar, within experimental error, to the quantum yield associated with the disappearance of HPT under similar conditions ($(2.0 \pm 0.3) \times 10^{-3}$), and to the quantum yield of H_2O_2 production in experiments performed under *aerobic* conditions ($(2.2 \pm 0.2) \times 10^{-3}$).

3. *The Role of the Superoxide Anion.* When air-equilibrated solutions containing 100 μM of HPT (**1a**) and 14 μM of cytochrome *c* (Cyt) at pH 6.7 were irradiated, an increase in the UV/VIS absorbance at 550 nm was observed. The difference in absorbance, ΔA_{550}, steadily increased with irradiation time, reaching a plateau after *ca.* 6 min (*Fig. 5*). The corresponding ED spectra in the range 500–600 nm were in good agreement with those reported in the literature for the reduction of Cyt [47]. Photoreduction of Cyt was not observed in similar experiments performed in the presence of superoxide dismutase (SOD; 200 U/ml), as can be seen from *Fig. 5*. These results show that, under the present conditions, $O_2^{\bullet -}$, generated photochemically by HPT, is the actual species that transfers an electron to Cyt.

When the same assay was carried out under anaerobic conditions, photoreduction of Cyt was also observed, even in the presence of SOD (200 U/ml; *Fig. 5*). Moreover, the extent of Cyt reduction was higher than in the presence of air, *i.e.*, the plateau was reached at a higher ΔA_{550} value. Thus, the reduction of Cyt, photosensitized by HPT (**1a**), can occur in the absence of O_2 by means of a direct electron transfer. The

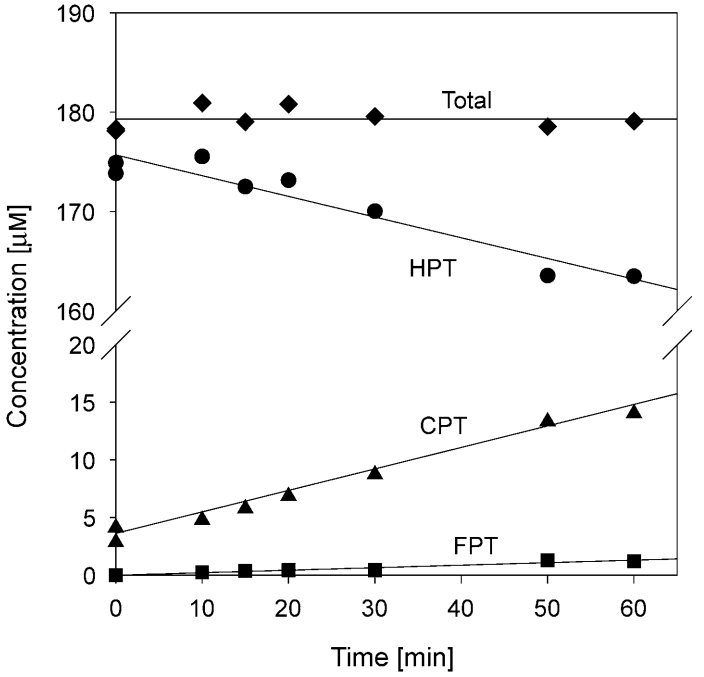


Fig. 4. *Evolution of the HPLC-based concentrations of HTP* (**1a**)*, FTP* (**5**)*, and CPT* (**6**). The initial concentration of **1a** was 180 μM. The aq. solutions (pH 5.5) were irradiated in the absence of O_2, and aerated in the dark immediately *after* irradiation. The mathematical sum (◆) of the different experimental concentrations is also shown.

photoreduction of Cyt in the absence of O_2 has been observed before for other heterocyclic compounds in aqueous solution [31].

Control experiments were performed to investigate thermal or photochemical processes that might interfere with the assay. No reduction of Cyt was observed in solutions containing 100 μM of **1a** and 14 μM of Cyt at pH 6.7, when kept in the dark for more than 2 h. When solutions containing only Cyt were exposed to UV-A radiation, no changes were observed in the spectra.

Taking into account the studies reported in the literature on the photooxidation of different pterin derivatives (see *Introduction* and *Scheme 2*), in combination with the results presented in the previous sections, it can be concluded that the intermediate(s) generated after irradiation of HPT (**1a**) is (are) able to transfer one electron either to O_2 to yield $O_2^{\bullet-}$, or directly to Cyt. In air-equilibrated solutions, where there is a large excess of O_2 over Cyt, the first process takes place. However, in the absence of O_2, the latter occurs. Thereby, the observed H_2O_2 is produced from $O_2^{\bullet-}$ by the following reactions:

$$H_3O^+ + O_2^{\bullet-} \rightleftharpoons HO_2^{\bullet} + H_2O \ (pK_a = 4.85\ [48]) \quad (3)$$

$$H_3O^+ + O_2^{\bullet-} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2 + H_2O \quad (4)$$

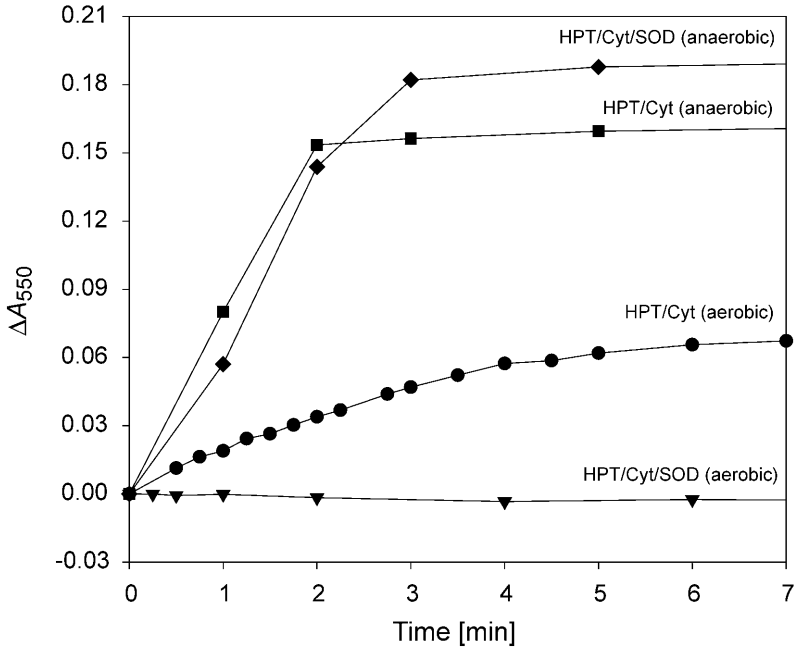


Fig. 5. *Photoreduction of cytochrome* c (Cyt; 14 μM) *sensitized with* **1a** (100 μM) *in aqueous solution* (pH 6.7). The experiment was performed in the presence and absence of O_2, and in the presence and absence of superoxide dismutase (SOD).

When taking into account the mechanism proposed in *Scheme 3* and the fact that $O_2^{\bullet-}$ can abstract H^+ from very weak acids, then one can conclude that the H-atoms in H_2O_2 may come either from H_2O (*Eqns. 3* and *4*) or from the pterinic intermediates.

4. *Singlet-Oxygen Studies.* The value of the apparent quantum yield of $^1O_2$ production, Φ_Δ^{app}, by the acid form **1a** of HPT was determined in O_2-saturated and air-equilibrated D_2O solutions by monitoring the near-IR $^1O_2$ luminescence (see *Experimental*). Most pterins give rise to a relatively high fluorescence quantum yield Φ_F [49]. Therefore, control experiments in Ar-saturated solutions were carried out to check possible tailing of the fluorescence emission of **1a** in the near-IR. However, no luminescence was detected at 1270 nm under the experimental conditions.

Experiments for determining Φ_Δ^{app} were performed at pD 5.5. Now, a significant $^1O_2$ emission was observed, and a value of 0.15 ± 0.02 was determined for air-equilibrated solutions. Taking into account the rate constant of $^1O_2$ total quenching by the basic form **1b** ($k_t = 3.1 \times 10^6$ M^{-1} s^{-1}) [23], and assuming that this value does not drastically change for the acidic form **1a**, Φ_Δ^{app} may be considered as Φ_Δ.

It is worth mentioning that the value of Φ_Δ^{app} for the acid form **1a** did not increase when the solutions were saturated with O_2. Thus, the excited triplet state was completely quenched by O_2 in the air-equilibrated solutions. The value of Φ_Δ^{app} obtained for the acid form **1a** of HPT (0.15 ± 0.02) is a little lower than that previously found for the corresponding basic form **1b** ($\Phi_\Delta = 0.21 \pm 0.01$) [23], an observation made before for other pterin derivatives [13].

Taking into consideration that the acidic form **1a** of HPT is able to generate $^1O_2$ under UV-A irradiation, it is necessary to check if this ROS is involved in the mechanism of the photoreaction of HPT. Thus, photolysis experiments were performed in D_2O. Air-equilibrated solutions of 260 μM of **1a** at pH 5.5 in H_2O and pD 5.5 in D_2O were irradiated, and UV/VIS spectra were recorded as a function of irradiation time under both experimental conditions. Spectroscopic analysis showed that the photooxidation of HPT (**1a**) to FPT (**5**) and CPT (**6**) is not accelerated in D_2O. This result indicates that $^1O_2$ does not actively participate in the main mechanism of photooxidation of HPT, in agreement with the results presented above.

5. *Mechanistic Considerations.* Taking into account the results discussed in the previous sections, we propose the reaction mechanism shown in *Scheme 3* (see above). We suggest that irradiation of the acid form **1a** of HPT gives rise to 6-formyl-5,8-dihydropterin (**4**) by means of a process similar to that already reported for the basic form **1b** [23]. The same holds for other pterin derivatives [22], where 6-acyl-5,8-dihydropterin intermediates were formed. However, under our specific experimental conditions, **4** might also yield another intermediate through tautomerization (keto/enol equilibrium). This alternative intermediate, the enol tautomer **4′** in *Scheme 3*, could then react with O_2 to yield CPT (**6**).

6. *Theoretical Considerations.* To compare the stability of HPT (**1a**) and the photoproducts **4** and **4′**, their ground-state geometries, heats of formation (ΔH_f), and other properties were calculated by means of the semiempirical parametrized PM3 method (*Table 1*). The loss of the aromatic character of the pterin moiety on going from **1a** to **4** and **4′** agrees with the different calculated ΔH_f values and the experimental observation that both **4** and **4′** are stable only under inert atmosphere, both being formed from the electronic excited state of **1a**.

Table 1. *Calculated Heats of Formation* (ΔH_f), *HOMO and LUMO Energies* (*E*), *Maximum-Absorption Wavelengths* (λ_{max}), *and Oscillator Strengths* (*f*) *of Selected Compounds*

	ΔH_f [kcal mol^{-1}]	E_{HOMO} [eV]	E_{LUMO} [eV]	ΔE (LUMO − HOMO) [eV]	λ_{max} [nm][a])	*f*
1a	−31.43	−9.29	−1.24	8.05	308	0.632
4	−30.34	−7.76	−0.85	6.91	366	0.011
4′	−20.82	−7.84	−0.95	6.89	393	0.226

[a]) Calculated λ_{max} values for **5** and **6**: 327 and 317 nm, resp.

The HOMO and LUMO energies were calculated to compare the relative reactivities of these three compounds towards O_2. As shown in *Table 1*, the energy of the HOMO orbital, E_{HOMO} of **4** and **4′** are higher than that of HPT (**1a**). Since the HOMO energy is related to nucleophilicity, this finding indicates that the two isomers **4** and **4′** are more nucleophilic than the starting material **1a**.

Next, the charge density at each C-atom was evaluated (*Table 2*). We found considerably higher charge densities for the C(6)=CH_2 moiety in **4** and **4′** compared to **1a**, indicating a higher nucleophilic character of this double bond and, thus, a higher reactivity towards O_2. Therefore, the C(α)−OOH (hydroperoxide) intermediate can be easily formed yielding CPT (**6**) as the final product.

Table 2. *PM3-Calculated Charge Densities for Atoms C(6) and C(α)*

Position	**1a**	**4**	**4′**	**5**	**6**
C(6)	−0.182	−0.271	−0.229	−0.255	−0.174
C(α)	0.133	0.335	−0.035	0.334	0.445

Finally, the UV/VIS absorption spectra of **1a**, **4**, and **4′** were simulated with the ZINDO/S method. A clear bathochromic shift of λ_{max} for **4** and **4′** was predicted, in agreement with the bathochromic shift experimentally observed during irradiation of **1a** under inert atmosphere.

Conclusions. – Photooxidation of the acid form **1a** of 6-(hydroxymethyl)pterin (HPT; *Scheme 1*) shows a different pattern to that observed in the photooxidation of HPT in alkaline medium (basic form **1b**) [23]. The quantum yields associated with the disappearance of **1a**, determined in the presence and in the absence of O_2, are similar (2.3×10^{-3}), but much lower than that determined for **1b** (1.8×10^{-2}) [23]. The photoreaction occurs through at least two steps. In the first, after absorption of UV-A radiation and without participation of O_2, two intermediates (**4** and **4′**) are generated (*Scheme 3*). In the second step, these intermediates react with O_2 to yield FPT (**5**) and CPT (**6**), the proportion of the latter being much higher than that of the former.

During photooxidation of HPT (**1a**), the superoxide anion ($O_2^{\bullet-}$) is generated, probably as a consequence of electron transfer from these intermediates to O_2 present in solution. This result is very important from a biological point of view, because $O_2^{\bullet-}$, along with other reactive oxygen species (ROS), is implicated in the etiology of many pathological conditions. To the best of our knowledge, the photoinduced generation of $O_2^{\bullet-}$ by oxidized pterins has not been reported before. One molecule of H_2O_2 is generated for each molecule of HPT consumed, H_2O_2 probably being a product of the thermal dismutation of $O_2^{\bullet-}$.

The acidic form **1a** of HPT is a singlet-oxygen ($^1O_2$) sensitizer under UV-A irradiation in aqueous solution ($\Phi_\Delta^{app}=0.15\pm0.02$). This result is in good agreement with previous studies reported on other pterin derivatives [12]. However, experiments in D_2O indicate that $^1O_2$ does not participate in the main pathway of the photooxidation of HPT to FPT (**5**) and CPT (**6**).

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