Generation of Reactive Oxygen Species during the Photolysis of 6-(Hydroxymethyl)pterin in Alkaline Aqueous Solutions

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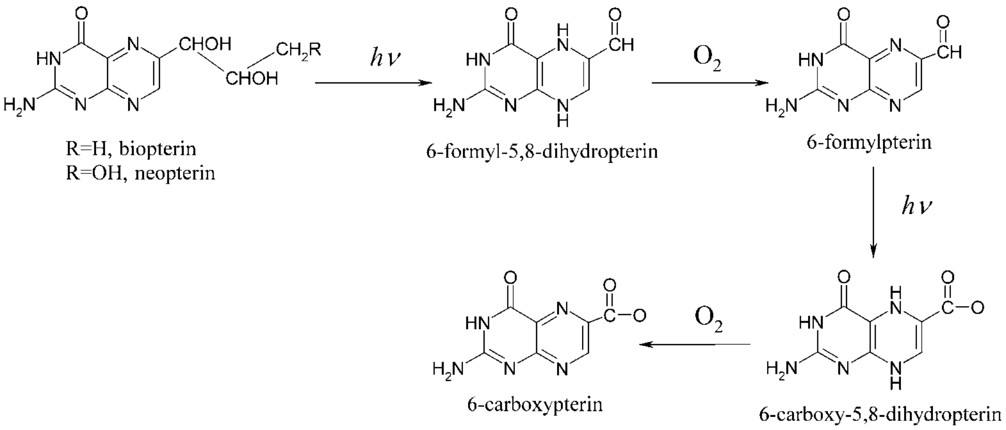
Photochemical studies of the reactivity of 6-(hydroxymethyl)pterin (=2-amino-6-(hydroxymethyl)pteridin-4(1*H*)-one; HPT) in alkaline aqueous solutions (pH 10.2–10.8) at 350 nm and room temperature were performed. The photochemical reactions were followed by UV/VIS spectrophotometry, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and an enzymatic method for H_2O_2 determination. In the presence of O_2, 6-formylpterin (=2-amino-3,4-dihydro-4-oxopteridine-6-carboxaldehyde; FPT) was the only photoproduct detected. In the absence of O_2, we observed a compound with an absorbance maximum at 480 nm, which was oxidized very rapidly by O_2 in a dark reaction to yield FPT. The quantum yields of substrates disappearance and of photoproducts formation were determined. The formation of H_2O_2 during photooxidation was monitored, and the number of mol of H_2O_2 released per mol of HPT consumed corresponded to a 1:1 stoichiometry. HPT was also investigated for efficiency of singlet-oxygen ($^1O_2$) production and quenching in aqueous solution. The quantum yield of $^1O_2$ production ($\Phi_\Delta = 0.21 \pm 0.01$) was determined by measurements of the $^1O_2$ luminescence in the near-IR (1270 nm) upon continuous excitation of the sensitizer. The rate constant of $^1O_2$ total quenching by HPT was determined ($k_t = 3.1 \cdot 10^6$ M^{-1} s^{-1}), indicating that this compound was able to quench $^1O_2$. However, $^1O_2$ did not participate in the photooxidation of HPT to FPT.

Introduction. – Pterins (=2-aminopteridin-4(1*H*)-ones) are a family of heterocyclic compounds [1][2] present in a wide variety of biological systems. Although they are present in small amounts in living systems, it is well-known that pterins participate in important biological functions [1–3]. The light sensitivity of pterins has been known since several decades [4][5], and interest in photochemistry and photophysics of this group of compounds has been shown since participation of pterins in different photobiological processes has been established or suggested. A derivative of folic acid, a conjugated pterin, is the light-harvesting chromophore of DNA photolyases [6][7], enzymes involved in DNA repair processes that take place after UV irradiation. It has been suggested that pterins may play some role in photosynthesis [8], and they have been found in photosensitive organs such as the eyes of different animals [9]. Pterins are also interesting as possible photosensitizers in photochemical processes that take place in organisms exposed to UV-A radiation; *e.g.*, pterins act as sensitizers in photochemical reactions that induce DNA damage [10][11] and are able to generate singlet oxygen ($^1O_2$) [12][13]. Some biomedical studies suggest that pterins are involved in pathogenesis of vitiligo, a depigmentation disorder [14][15]. Moreover, the accumulation of 6- and 7-biopterin in the epidermis of the affected patients leads to a typical fluorescence under *Wood*'s light (351 nm) [14].

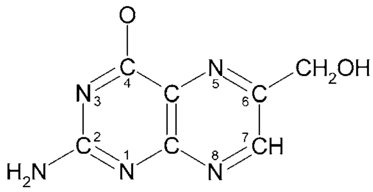
Pterins behave as weak acids in aqueous solution. As reported by *Albert* [16] for several pterin derivatives, the dominant equilibrium at pH higher than 5 involves an amide group (acid form) and a phenolate group (basic form). The pK_a of this equilibrium is *ca.* 8 for the different pterin derivatives studied [16–19]. Other functional groups of the pterin moiety (*e.g.*, the 2-amino group or ring N-atoms) have pK_a values lower than 2 [16].

The 6-substituted pterins, the most-common pterin derivatives found in nature, undergo photooxidation in air-equilibrated aqueous solutions [19–25]. Thus, it has been reported that the mechanism involved in the photochemical transformation of these compounds is considerably affected by the nature of the substituent at position 6 of the pterin moiety, and that the photochemistry of the acidic and basic forms of the pterin derivatives are different. Our previous studies suggested that $^1O_2$ participates in photooxidation of folic acid [19][20], whereas, in the mechanism involved in the photooxidations of biopterin and neopterin studied by *Pfleiderer* and co-workers [24][25], $^1O_2$ does not participate. On the contrary, an intermediate generated in an O_2-independent process (*Scheme 1*) is thermally oxidized on introduction of O_2. The mechanism of the photooxidation of 6-formylpterin (=2-amino-3,4-dihydro-4-oxopteridine-6-carboxaldehyde; FPT) to yield 6-carboxypterin (=2-amino-3,4-dihydro-4-oxopteridine-6-carboxylic acid; CPT) [19][21] (*Scheme 1*) is similar to that described for biopterin and neopterin. Finally, photochemical decomposition of CPT in the presence of O_2 [22] occurs by two main reaction pathways: decarboxylation to yield pterin (this photochemical process is O_2-independent and occurs only for the acid form), and photooxidation of the pterin moiety to yield non-pterinic photoproducts.

Scheme 1. *Photooxidation of Biopterin, Neopterin* [24][25], *and 6-Formylpterin* [19][20]



In the present paper, we describe the photochemistry of 6-(hydroxymethyl)pterin (=2-amino-6-(hydroxymethyl)pteridin-4-(1*H*)-one; HPT) in alkaline aqueous solution where only the basic form of the molecule is present. Photoproducts were identified. The quantum yield of HPT disappearance and that of the formation of photoproducts were determined. In addition, the role of O_2 in the mechanisms of these photochemical reactions was analyzed, and the capability of HPT to yield activated oxygen species is discussed. In particular, we investigated and quantified the efficiency of $^1O_2$ production (Φ_Δ) and quenching by HPT. We also studied the production of H_2O_2 during the photooxidation of HPT. The results obtained in this study were compared with those described for the photochemistry of other pterin derivatives.



Experimental. – 1. *Chemicals.* The 6-(hydroxymethyl)pterin (HPT), 6-formylpterin (FPT), and other pterins were purchased from *Schircks Laboratories*, Switzerland, and used without further purification. Propan-2-ol and EtOH were purchased from *Merck* and other chemicals from *Sigma-Aldrich* and used as received. The pH of the aq. solns. was adjusted by adding drops of NaOH solns. from a micropipette. The concentration of the base used for this purpose ranged from 0.1 to 2M. The ionic strength was *ca.* 10^{-3} M in all the experiments. In singlet oxygen ($^1O_2$) experiments, D_2O (*Euriso-top*, *Groupe CEA*, Saclay, France; minimum isotopic purity of 99.9%), DCl (*Aldrich*, 99.5% D) soln. in D_2O and NaOD (*CEA*) soln. in D_2O were employed.

2. *Determination of* pK_a. pK_a Values were determined from absorption changes. Measurements were performed at r.t. The experimental absorption changes at a given wavelength can be fitted by *Eqn. 1*, where ε_a and ε_b are the molar absorption coefficients of the acid and basic forms of the species involved in the acid-base equilibrium, c is the total concentration of the substance, l is the optical length, and K_a is the dissociation constant. A more-detailed description of pK_a determinations has been described elsewhere [17][18].

$$A = (c \cdot l) \cdot \{\varepsilon_a + (\varepsilon_b - \varepsilon_a) \cdot [K_a/(K_a + [H^+])]\} \qquad (1)$$

3. *Continuous photolysis.* 3.1. *UV Irradiation.* The continuous photolysis of HPT solns. were carried out in quartz cells (1-cm path length) at r.t. *Rayonet-RPR* lamps emitting at 350 nm (*Southern N.E. Ultraviolet Co.*) were employed for irradiating. Photolysis experiments were performed in the presence and in the absence of air. Deaerated solns. were obtained by bubbling with Ar for 20 min.

3.2. *UV/VIS Spectrophotometric Analysis.* A *Cary-3* spectrophotometer (*Varian*) and a program for smoothing and averaging the signals of the spectra were used. The irradiations were performed in quartz cells of 1-cm optical-path length, and the absorbance measurements were made in quartz cells of 1-cm and 0.2-cm optical-path length. Absorption spectra of the solns. were recorded at regular intervals of irradiation time.

Experimental difference (ED) spectra were obtained by subtracting the spectrum at time $t = 0$ from the subsequent spectra recorded at different times. Each ED spectrum was normalized relative to the maximum absolute 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 characterization of the major photolysis products. The analysis based on these difference spectra is described elsewhere [19][22].

4. *Thin-Layer Chromatography* (TLC). DEAE-cellulose plates (100 μm) and 0.3% (*w*/*v*) aq. NH_4Cl soln. as eluent were used. Irradiated HPT solns. ($1.5 \cdot 10^{-4}$ M) and aq. solns. of pterin-derivatives standards were developed by exposing them to 350-nm radiation. Under these conditions, pterin derivatives show blue fluorescent spots. The corresponding R_f values obtained for pterin and several pterin derivatives have already been reported [19].

5. *High-Performance Liquid Chromatography* (HPLC). A *Konic Instruments 500-B* HPLC with a *C18-Spherisorb-S5-ODS2* (250 × 46 mm) column and a *Lambda-1000 Bischoff* HPLC spectrophotometer as detector was employed for the reaction monitoring, the identification of photoproducts, and for the quantification of HPT and photoproducts. A 5% MeCN/H_2O 60 : 40, 95% aq. soln. of 20 mM potassium phosphate (pH 5.5), and 2.5 mM EDTA were used as eluent. For irradiated HPT solns., HPLC runs were monitored at 340 nm.

6. *Quantum-Yield Determinations.* The quantum yields of HPT disappearance and FPT formation were determined in experiments performed under different conditions. *Aberchrome 540* was the actinometer employed for the measurements of photon rates [26] ($(7.2 \pm 0.3) \cdot 10^{-4}$ Einstein $\cdot$ $l^{-1} \cdot min^{-1}$). The initial concentration of the reactant was adjusted so that more than 99.99% of the incident radiation at 350 nm was absorbed. The evolution of the concentrations of reactants and photoproducts during the irradiation time was followed by HPLC. Aq. solns. of commercial standards were employed for obtaining the corresponding calibration curves.

7. *Determination of O_2 Concentration.* The O_2 consumption during the photolysis was measured with an O_2 selective electrode (*Orion*, model *37-08-99*). The experimental set-up for these measurements was described elsewhere [27].

8. *H_2O_2 Determination.* For determination of H_2O_2 the cholesterol kit from *Wiener* was used. H_2O_2 was quantified by its color reaction with 4-aminophenazone and phenol [28][29]. The irradiated HPT soln. (400 μl) was added to 2 ml of reagent. The absorbance at 505 nm of the resulting soln. was measured, after 30 min of incubation at r.t., by using the reagent as a blank. Aq. solns. of H_2O_2, prepared from commercial standards, were employed for obtaining the corresponding calibration curves.

9. *Hydroxy-Radical Quenching.* HPT Solns. (350 μM) were irradiated in the presence of propan-2-ol or EtOH. The concentrations of the alcohols ranged from 3.5 to 350 mM. These compounds have a high reactivity with hydroxy radicals ($HO^•$) [30] diminishing its concentration during the experiment. Results of UV/VIS spectrophotometric analysis were compared with those obtained in the absence of the selective $HO^•$ scavenger.

10. *Singlet Oxygen* ($^1O_2$) *Studies.* 10.1. *Determination of Quantum Yields of $^1O_2$ Production.* Quantum yield of $^1O_2$ production (Φ_Δ) was determined by direct analysis of the weak $^1O_2$ near-IR luminescence at 1270 nm (see, *e.g.* [31–33]), produced during continuous irradiation of the aq. solns. of HPT. The main features of the method and the equipment have already been described in detail [34–36]. In the present experiments, a cooled NIR photomultiplier (*Hamamatsu*) as detector, instead of a Ge photodiode, was used. Briefly, the sample soln. in a quartz cuvette was irradiated with a Xe/Hg arc through a H_2O filter, focusing optics, and a monochromator. The $^1O_2$ luminescence was collected with a mirror, chopped, and, after passing through focusing lens, a cut-off filter (1000 nm), and an interference filter (1271 nm), was detected at 90° with respect to the incident beam by using the cooled NIR photomultiplier. For determining Φ_Δ, the luminescence signals of a sensitizer soln. (S_e^S) and a soln. of a reference sensitizer (S_e^R) were measured. If the absorbances at the wavelength of irradiation are the same for the reference sensitizer and the sample solution, the ratio of the luminescence signals, S_e^S/S_e^R is given by *Eqn. 2*, where P_0^S/P_0^R is the ratio of the incident photon rates at the wavelength of excitation of the sensitizer investigated and of the reference. If k_d (the nonradiative-deactivation rate constant) and k_t^S (the rate constant of total (physical and chemical) $^1O_2$ quenching by the sensitizer itself) are known in the solvent used, 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 rates. Because of the short lifetime of $^1O_2$ ($\tau_\Delta = 1/k_d$) in H_2O (3.8 μs), D_2O was used as solvent in all experiments where τ_Δ is much longer (62 μs) [37][38]. Measurements with HPT were carried out at an excitation wavelength (λ_{ex}) of 367 nm. Rose bengal (RB) in D_2O (λ_{ex} 547 nm, $\Phi_\Delta^R = 0.75$ [39][40]) was used as a reference sensitizer.

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

10.2. *Determination of Rate Constants of Total $^1O_2$ Quenching by Pterins.* The rate constants of total $^1O_2$ quenching (k_t) by HPT was determined by *Stern–Volmer* analysis of the $^1O_2$-luminescence quenching. The main features of the method was described elsewhere [13][41]. $^1O_2$ was generated by photosensitization with RB as sensitizer. Groups of experiments were carried out in which solns. of HPT and RB were irradiated at 547 nm, where the investigated compound does not absorb. The RB concentration was kept constant, whereas the pterin-derivative concentration was varied within a series of experiments. Under our experimental conditions [13], a linear relationship between the ratio of the signals observed in the absence (S_e^0) and in the presence (S_e) of quencher (Q = HPT) and the quencher concentration is observed (*Eqn. 3*), where τ_Δ is the $^1O_2$ lifetime in the solvent used (D_2O) in the absence of Q. Therefore, knowing τ_Δ, k_t can be calculated from the slope of the *Stern–Volmer* plot.

$$S_e^0/S_e = 1 + k_t \tau_\Delta [Q] \tag{3}$$

11. *Continuous Photolysis in D_2O.* Solns. of HPT were prepared in H_2O and D_2O. Couples of both types of solns. containing HPT at the same concentration were irradiated under identical experimental conditions. The effect of D_2O was evaluated by comparing results of UV/VIS spectrophotometric analysis.

12. *Calculation of Molecular Properties.* The ground-state geometry, heat of formation, and HOMO and LUMO energies of HPT and 6-formyl-5,8-dihydropterin were calculated by using the semiempirical parametrized PM3 method as implemented in the 2002 version of the HyperChem program [42], which has been shown to be effective in studies of molecules containing heteroatoms, compared with other methods such as MINDO/3 or MNDO.

Results and Discussion. – *Determination of* pK_a *of 6-(Hydroxymethyl)pterin.* To the best of our knowledge, the pK_a value of the amide group in 6-(hydroxymethyl)pterin (HPT) has not been reported in the literature. We determined this pK_a, and a value of 8.1 ± 0.1 was found. This value is quite similar to those previously determined for other related pterin derivatives (pK_a(pterin) = 7.9 [17], pK_a(6-formylpterin) = 7.3 [19], and pK_a(6-carboxypterin) = 7.9 [17]). Taking into account the pK_a of HPT, we chose to use HPT solutions with $pH > 10$, so that only the basic form of HPT was present in the stock solutions.

Photolysis in the Presence of Oxygen. The spectrum of air-equilibrated solutions of HPT changes significantly with irradiation time, as shown in *Fig. 1,a*. An isosbestic point at 268 nm is observed during at least 12 min of irradiation. No further changes are detected in irradiated solutions that were stored in the dark. ED and NED spectra were obtained as described in the experimental section. The NED spectrum is similar to the NRD spectrum, obtained from standard solutions of HPT and 6-formylpterin (*Fig. 1,b*). This result suggests that under these experimental conditions, a new absorbing species with UV-absorption spectrum similar to that of FPT is formed. At irradiation times higher than 15 min, the NED spectrum changes and the isosbestic point at 268 nm disappears.

TLC Analysis shows the presence of three fluorescent substances in irradiated HPT solutions ($t = 0$, 10, 30, and 55 min). The obtained R_f values correspond to HPT, FPT, and 6-carboxypterin (CPT). Experiments performed at different irradiation times reveal that CPT appears at a later stage than FPT. This is in good agreement with previous results reported on photochemistry of FPT in alkaline aqueous solutions [21], *i.e.*, in the presence of O_2, FPT undergoes photooxidation to yield CPT.

In a similar way, the presence of FPT and CPT in irradiated solutions of HPT is confirmed by HPLC experiments. The HPT, FPT, and CPT concentrations were measured as a function of irradiation time by means of two sets of HPLC experiments. In the first group of experiments, concentrated solutions of HPT (350 μM) were irradiated. In the second group, irradiations were performed with solutions containing a mixture of HPT and FPT.

The first group of experiments shows that during the first 5 min of irradiation, the evolutions of the concentrations of HPT and FPT follow a zero-order rate law (*Fig. 2*). The rate of degradation of HPT is 13.7 ± 0.8 μM/min, whereas the rate of formation of FPT is 12.9 ± 0.9 μM/min. In this first stage, no significant increase in CPT concentration is observed. Thus, HPT seems to absorb the whole incident radiation in this time range. The quantum yield associated with the disappearance of HPT is $1.8 \cdot 10^{-2}$. As a value of $1.6 \cdot 10^{-2}$ is obtained for the quantum yield of FPT formation, the consumption of HPT to yield FPT would be the only process that takes place in the analyzed time range.

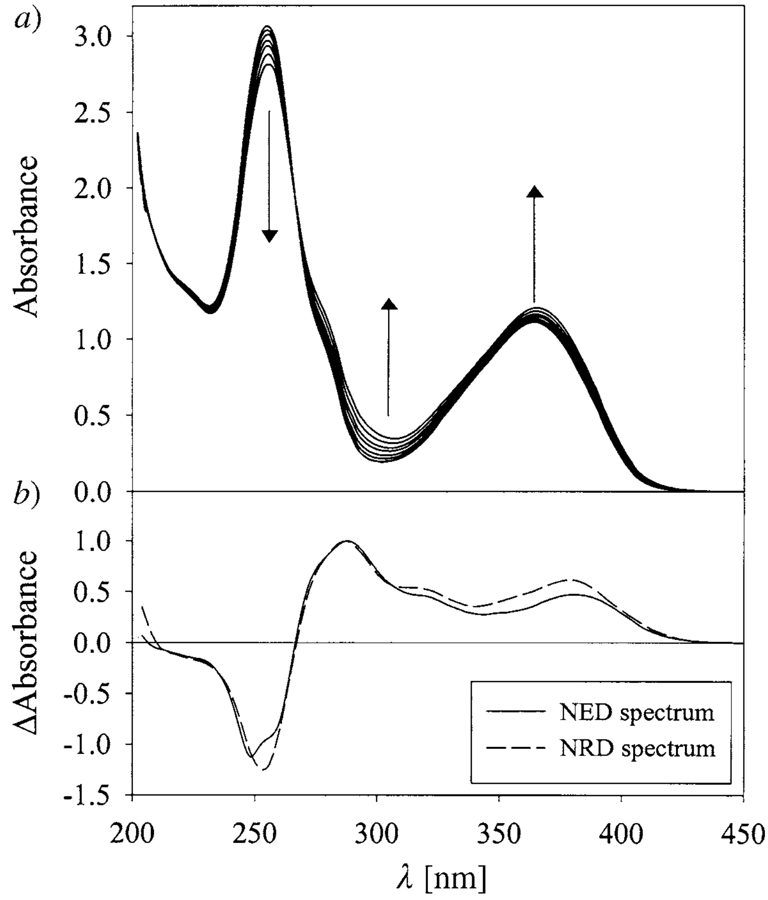


Fig. 1. a) *Evolution of the absorption spectra of irradiated solutions of 6-(hydroxymethyl)pterin* (150 μM at pH 10.5) *as a function of time* (experiment performed in the presence of O_2, spectra recorded every 2 min; arrows indicate the changes observed at different wavelengths). b) *NED Spectrum* (——) *obtained by subtracting the initial 6-(hydroxymethyl)pterin spectrum from the corresponding spectrum after 14 min of photolysis; NRD spectrum* (– – –) *obtained by subtracting the spectrum of a standard solution of 6-(hydroxymethyl)pterin from the spectrum of a standard solution of 6-formylpterin, at the same concentration and pH.*

After 5 min of irradiation, important increases in the rate of disappearance of HPT and in the rate of formation of FPT are observed (see *Fig. 3*). These changes coincide temporally with an increase of CPT concentration. As mentioned above, CPT is a photoproduct generated from FPT [21]. Therefore, after *ca.* 5 min, some significant proportion of radiation is absorbed by FPT. Thus, the chemical transformation from HPT to FPT seems to be enhanced through the participation of excited FPT. The formation of an FPT–HPT exciplex could be the cause of the enhancement observed. As can be seen in *Fig. 3*, during the first 20 min of irradiation, the total concentration of pterins (sum of HPT, FPT, and CPT concentrations) is almost constant, indicating that FPT and CPT are the main photoproducts.

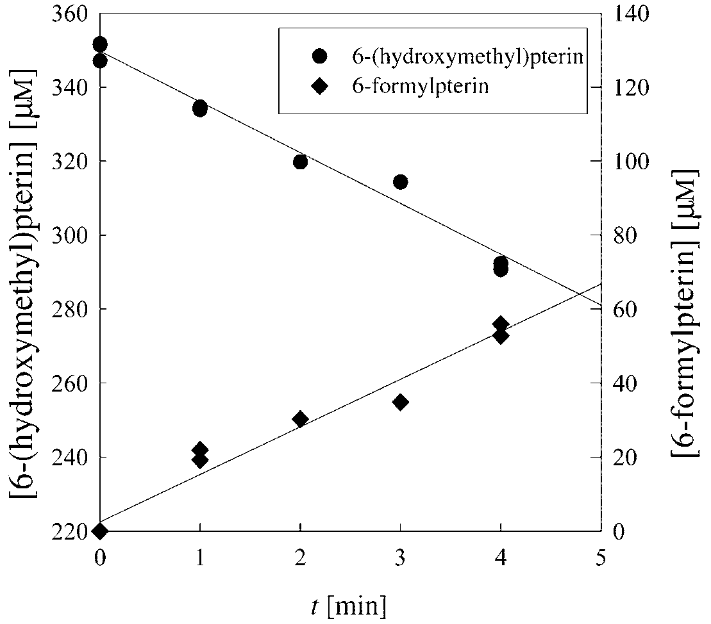


Fig. 2. *Evolution of 6-(hydroxymethyl)pterin and 6-formylpterin concentrations in an irradiated solution of 6-(hydroxymethyl)pterin* (350 µM at pH 10.5) *as a function of irradiation time.* HPLC Analysis during the first 4 min of photolysis.

In the second group of experiments, HPT absorbs a low proportion of the initial incident radiation, whereas a higher proportion is absorbed by FPT. As shown in *Fig. 4,a* solutions prepared as a mixture of 47 µM HPT and 315 µM FPT solutions were irradiated under air or He-atmosphere. Taking into account the quantum yield previously calculated ($1.8 \cdot 10^{-2}$) and that the rate of photons absorbed by HPT is 8% of the incident photon rate, a theoretical rate of consumption of HPT in the irradiated mixture can be estimated (– 1.2 µM/min). However, an experimental rate of – 5.3 ± 0.3 µM/min is obtained for the first 4 min of photolysis of the mixture in the presence of O_2. These results suggest that the absorption of radiation by FPT induces the conversion of HPT. As shown in *Fig. 4,b* similar results are obtained with solutions prepared as a mixture of 200 µM HPT and 310 µM FPT solutions.

The evolution of the O_2 concentration during irradiation was monitored with an O_2 electrode in a closed cell. As shown in *Fig. 5*, O_2 concentration decreases with the irradiation time. Moreover, after a few minutes of irradiation, an increase on the rate of consumption of O_2 is observed. The degradation of HPT induced by FPT and the photooxidation of FPT itself may explain this observation.

H_2O_2 was detected in irradiated solutions of HPT, indicating that photooxidation of HPT generates this reactive oxygen species. Concentration of H_2O_2 was determined at different times during the irradiation of a HPT solution (360 µM, pH 10.2) (*Fig. 6*). The estimated rate of production of H_2O_2 is 14 ± 2 µM/min in the first 4 min of irradiation. The quantum yield of H_2O_2 formation is calculated from that rate value and taking into

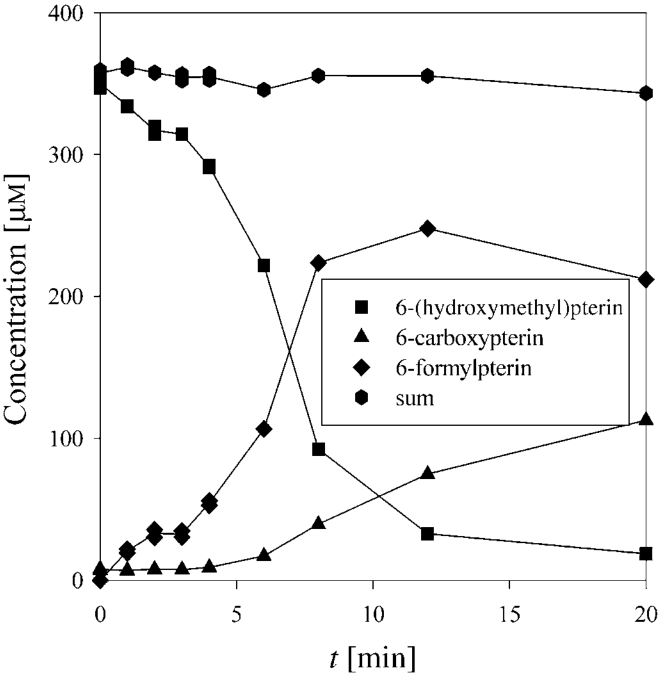


Fig. 3. *Evolution of 6-(hydroxymethyl)pterin, 6-formylpterin, and 6-carboxypterin concentrations in an irradiated solution of 6-(hydroxymethyl)pterin* (350 µM at pH 10.5) *as a function of time.* HPLC Analysis during 20 min of photolysis; (●) sum of the 6-(hydroxymethyl)pterin, 6-formylpterin, and 6-carboxypterin concentrations in µM, at each time.

account the incident photon rate (see details in the *Exper. Part*). Thus a value of $1.7 \cdot 10^{-2}$ is obtained. The comparison of the quantum yield of H_2O_2 formation and the quantum yields of HPT consumption and FPT formation suggest that for each molecule of HPT consumed, one molecule of H_2O_2 is generated.

Generation of H_2O_2 during the photooxidation of other pterin derivatives (biopterin, sepiapterin, and FPT) has previously been reported [15]. Generation of H_2O_2 in photolysis of pterins is important from a biomedical point of view and is particularly relevant for skin diseases. Thus, H_2O_2 participates in the pathogenesis of vitiligo [43], and the accumulation of pterins in the skin of patients that suffer that disease has been established [15][44].

To investigate whether the hydroxyl radical ($HO^{\bullet}$) is involved in the mechanism of photooxidation of HPT, photolysis in the presence of propan-2-ol or EtOH, selective $HO^{\bullet}$ scavengers [30], were carried out. HPT Solutions (350 µM, pH 10.5) were irradiated in the presence of different concentrations of alcohol (3.5–350 mM). Absorption spectra of the solutions were recorded at regular intervals of irradiation time during 15 min, and ED and NED spectra were obtained. These results were compared with those obtained in the absence of the selective $HO^{\bullet}$ scavenger, and no significant differences are detected, thus suggesting that $OH^{\bullet}$ does not participate in the photochemical processes studied.

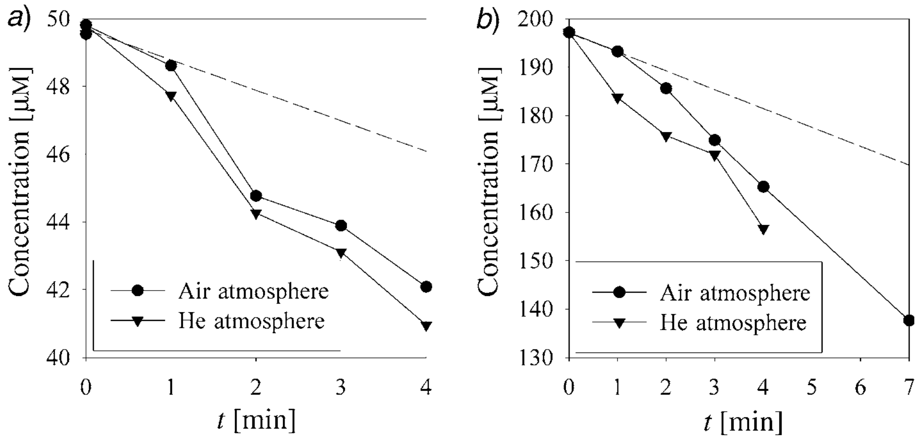


Fig. 4. *Evolution of 6-(hydroxymethyl)pterin concentration in irradiated solutions containing a mixture of 6-(hydroxymethyl)pterin and 6-formylpterin as a function of time:* a) *47* μM *6-(hydroxymethyl)pterin and 315* μM *6-formylpterin* (pH 10.5); b) *200* μM *6-(hydroxymethyl)pterin and 310* μM *6-formylpterin* (pH 10.5). HPLC Analysis; the experiments were performed in the presence and in the absence of O_2; – – – = theoretical rate of consumption of 6-(hydroxymethyl)pterin, estimated taking into account the intensity of the incident photon rate absorbed by 6-(hydroxymethyl)pterin and the quantum yield of consumption calculated in the absence of 6-formylpterin.

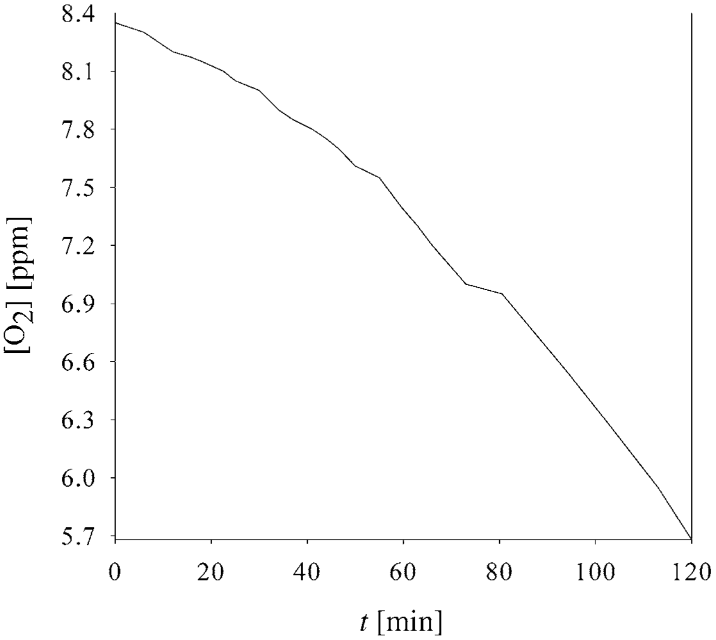


Fig. 5. *Evolution of the O_2 concentration in an irradiated solution of 6-(hydroxymethyl)pterin* (150 μM at pH 10.2) *as a function of irradiation time*

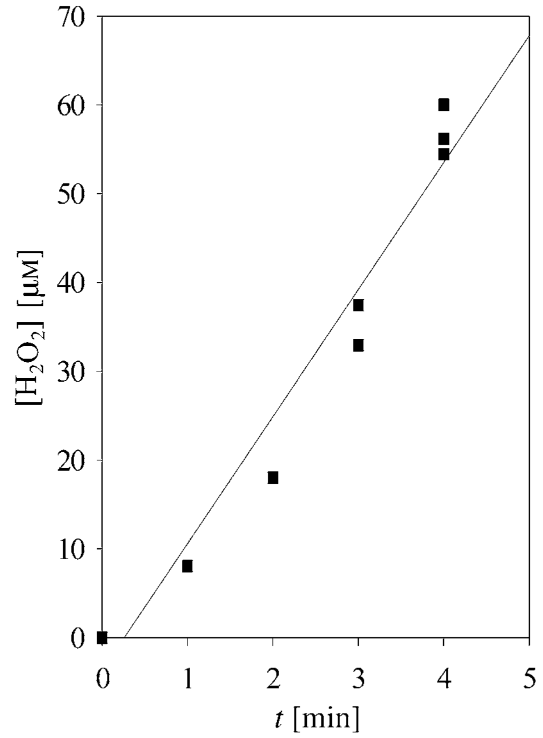


Fig. 6. *Evolution of the H_2O_2 concentration in an irradiated solution of 6-(hydroxymethyl)pterin* (360 μM at pH 10.2) *as a function of irradiation time*

Moreover, the concentration of H_2O_2 was determined at different times during the irradiation of a HPT solution (350 μM, pH 10.2) in the presence of propan-2-ol (350 mM). The rate of H_2O_2 production is 11 ± 2 μM/min in the first 4 min of irradiation. This rate value is similar, within the experimental error, to that obtained under the same experimental conditions but in the absence of the $HO^{\bullet}$ scavenger (14 ± 2 μM/min). These results indicate that $HO^{\bullet}$ is not involved in the oxidative process that yields H_2O_2.

Photolysis in the Absence of Oxygen. To elucidate the role of O_2 in the photochemistry of HPT, solutions previously bubbled with Ar were irradiated. Under these conditions, visible changes in the color of the solutions were observed. As shown in *Fig. 7*, anaerobic photolysis of HPT leads to the formation of a compound with a longwave absorbing band of low molar absorption coefficient at 480 nm. This compound will be named throughout as 'red intermediate' (see its possible structure in *Scheme 2*, below). Commercially available pterin-derivative standards do not present such spectrophotometrical characteristics and, therefore, the NED spectrum of this photolysis does not agree with the NRD spectra obtained from commercial standard solutions.

The 'red intermediate' obtained in O_2-free solutions suffers a thermal reaction in the dark as soon as air or O_2 is admitted into the cell. The band around 480 nm

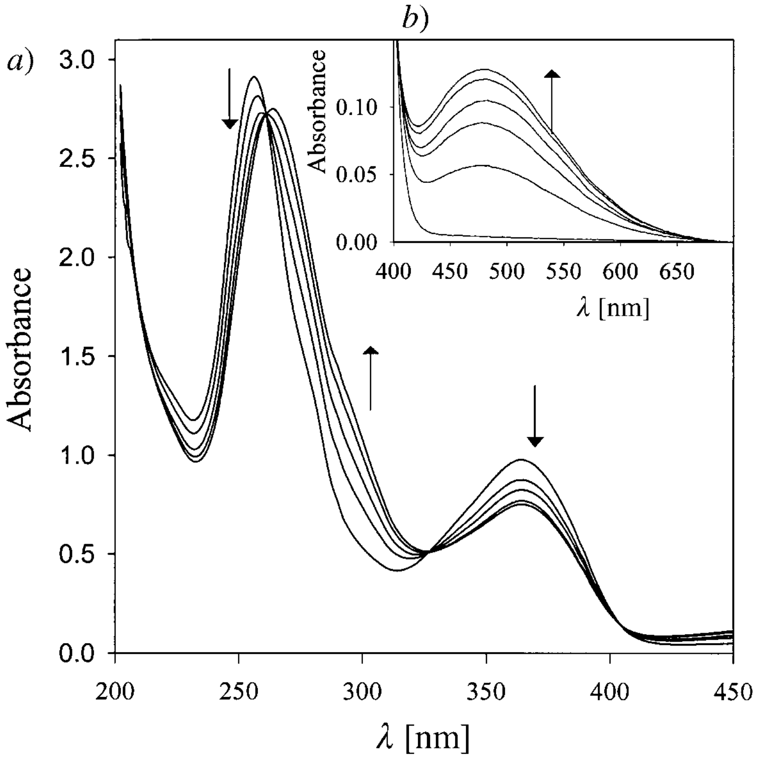
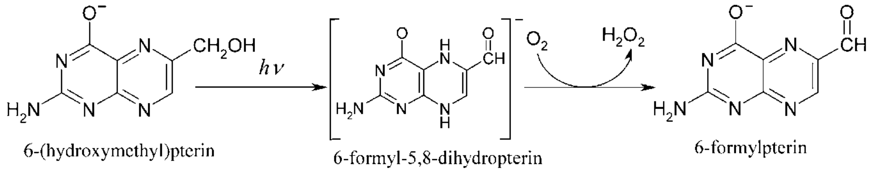


Fig. 7. *Evolution of the absorption spectra of irradiated solutions of 6-(hydroxymethyl)pterin* (150 μM at pH 10.1) *as a function of time.* Experiment performed in the absence of O_2; spectra were recorded every 10 min, arrows indicate the changes observed at different wavelengths.

Scheme 2. *Photooxidation of 6-(Hydroxymethyl)pterin in Alkaline Aqueous Solutions* (pH 10.0–10.5) *upon Irradiation with UV-A Radiation* (*λ* 350 nm)



disappears. The NED spectrum, obtained by subtracting the spectrum of a non-irradiated solution of HPT from that of an irradiated and then immediately aerated solution, matches with the NRD spectrum obtained from standard solutions of HPT and FPT. This fact suggests that an FPT-like chromophore is the final oxidized stable structure of the 'red intermediate' (*Scheme 2*).

TLC Analysis of the irradiated HPT solutions under anaerobic conditions and then aerated show a fluorescent spot having an R_f identical to that of FPT. The intensity of the spot of HPT decreases with the irradiation time. No other product could be detected by this procedure.

The results obtained by HPLC analysis are in good agreement with those obtained by TLC, *i.e.*, FPT is the only photoproduct found in HPT solutions irradiated in the absence of O_2 and then immediately aerated. In the first 4 min of irradiation the rate of consumption of HPT is 13.6 ± 1.5 µM/min (quantum yield of $1.8 \cdot 10^{-2}$), whereas the rate of formation of FPT is 12.5 ± 1.2 µM/min (quantum yield of $1.8 \cdot 10^{-2}$). The quantum yield obtained under these experimental conditions is equal to that measured in the presence of O_2 ($1.8 \cdot 10^{-2}$). *Fig. 8* shows that no increase in the rate of photodisappearance of HPT and of the rate of formation of FPT is observed during 20 min of photolysis, confirming that the acceleration of the photolysis observed in the presence of O_2 is not due to the accumulation of the 'red intermediate'. On the contrary, accumulation of some reactive intermediate formed during the photolysis of FPT could be the cause of the observed acceleration.

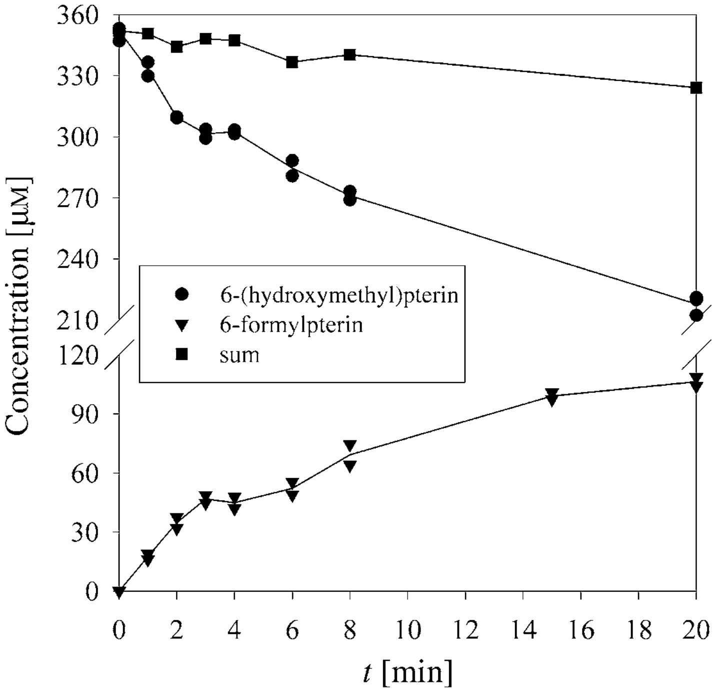


Fig. 8. *Evolution of 6-(hydroxymethyl)pterin and 6-formylpterin concentrations in an irradiated solution of 6-(hydroxymethyl)pterin* (350 µM at pH 10.5) *as a function of time.* Experiment performed in the absence of O_2; HPLC analysis during 20 min of photolysis; ■ = sum of 6-(hydroxymethyl)pterin and 6-formylpterin concentrations in µM, at each irradiation time.

Measurements of H_2O_2 in solutions of HPT (360 µM, pH 10.2) immediately aerated after different times of irradiation in anaerobic conditions reveal that H_2O_2 is generated under these experimental conditions. The value obtained for the rate of H_2O_2 production is 15 ± 1 μM/min for the first 3 min of irradiation. This result suggests that H_2O_2 is a product of the thermal reaction between the 'red intermediate' and O_2.

The 'red intermediate', detected in the absence of O_2, seems to participate in an active way in the oxidation of HPT to FPT. The similar quantum yields measured in the presence and the absence of O_2 support this hypothesis. Under air, the 'red intermediate' oxidizes immediately to FPT, explaining why the 'red intermediate' can be detected only under anaerobic conditions. If this is the reaction pathway, $^1O_2$ is not necessary in the later step of the formation of FPT from HPT, because the yield of FPT from the 'red intermediate' takes place in the absence of UV radiation. The role of $^1O_2$ will be analyzed in detail in the next section.

The mechanism involved in the photolysis of HPT seems to be similar to that found in photooxidation of biopterin, neopterin [24] [25] and FPT [19] [21] (see *Introduction*). It has been proposed that the intermediates detected in these photooxidations, which have spectral features and reactivities similar to those of the so-called 'red intermediate', are 6-acyl-5,8-dihydropterins. Therefore, the 'red intermediate' could be a 6-formyl-5,8-dihydropterin, one of the isomeric forms of HPT (*Scheme 2*), which is easily formed in the absence of O_2 from the electronically excited HPT. Further investigations to obtain more evidence on this are in progress in our laboratory.

To compare the stability of HPT and its isomer, 6-formyl-5,8-dihydropterin, their ground-state geometry, and heat of formation (ΔH_f) were calculated by using the semiempirical parameterized PM3 method. The loss of the aromatic character of the pterin moiety on going from the former to the latter structure (see *Scheme 2*) agrees with the different calculated ΔH_f and the experimental finding that the 6-formyl-5,8-dihydropterin is stable only under inert atmosphere conditions, and it is formed from the electronically excited state of HPT.

The molecular-orbital energies were computed to compare the relative reactivity of HPT and of its isomer, the 6-formyl-5,8-dihydropterin, with O_2. As shown in the *Table*, on going from HPT to its isomer, the difference calculated between the HOMO and LUMO levels is in between 7.70 and 6.42 eV, and clearly the energy of the highest occupied molecular orbital (HOMO) increases from −4.50 to −3.70 eV. Since the energy of the HOMO is regarded as a criterion of nucleophilicity, this finding indicates that the 6-formyl-5,8-dihydropterin has the highest nucleophilicity among the two isomers.

Table. *Calculated* ΔH_f *and HOMO and LUMO Energy*

	ΔH_f/kcal mol^{-1}	HOMO/eV	LUMO/eV	Δ (LUMO – HOMO)/eV
6-(Hydroxymethyl)pterin[a])	−81.98	−4.51	3.19	7.70
6-Formyl-5,8-dihydropterin[a])	−75.11	−3.70	2.73	6.42

[a]) Anionic form, net charge −1 (see *Scheme 2*)

The Role of Singlet Oxygen ($^1O_2$). The value of the quantum yields of $^1O_2$ production (Φ_Δ) by HPT was determined in O_2-saturated and air-equilibrated D_2O solutions, by monitoring the near-IR $^1O_2$ luminescence. Most pterins have a relatively high fluorescence quantum yield (Φ_F) [45]. Therefore, control experiments in Ar-saturated solutions were carried out to check possible tailing of the fluorescence emission of HPT in the near-IR. No luminescence at 1270 nm can be detected under those experimental conditions.

Experiments for determining Φ_Δ were performed at pD 10.5. Significant $^1O_2$ emission is detected, and a value of 0.21 ± 0.01 is obtained in air-equilibrated solutions. No significant differences in Φ_Δ values are observed in experiments carried out in O_2-saturated solutions. The value of Φ_Δ obtained for HPT is a little lower than those previously found for FPT, CPT, and pterin [13] under similar experimental conditions.

$^1O_2$ relaxes to ground-state $^3O_2$ by both radiationless and radiative pathways (*Eqns. 4* and *5*, resp.).

If a substance in solution is able to quench or trap $^1O_2$, chemical reaction (*Eqn. 6*) and physical quenching (*Eqn. 7*) must be considered.

$$^1O_2 \xrightarrow{k_d} {}^3O_2 \tag{4}$$

$$^1O_2 \xrightarrow{k_e} {}^3O_2 + h\nu' \tag{5}$$

$$Q + {}^1O_2 \xrightarrow{k_r} QO_2 \tag{6}$$

$$Q + {}^1O_2 \xrightarrow{k_q} Q + {}^3O_2 \tag{7}$$

The values of the rate constants of total $^1O_2$ quenching ($k_t = k_r + k_q$) by HPT were determined as indicated in the *Exper. Part*. *Fig. 9* reveals that the *Stern-Volmer* plots of the quenching of the near-IR $^1O_2$ luminescence (*Eqn. 3*) are linear within the range of concentrations used. The value of k_t is calculated from the slope of this plot, taking a value of 62 μs for the $^1O_2$ lifetime (τ_Δ) in D_2O [37][38]. Under our experimental conditions, a value of $3.1 \cdot 10^6$ M^{-1} s^{-1} is calculated. It is important to take into account that this value is similar to those previously reported for pterin and FPT [13] as well as that $^1O_2$ is not involved in the mechanisms of their photooxidations [19][21]. On the other hand, this value is significantly lower than that reported for folic acid [13], where $^1O_2$ seems to be involved in the photooxidation pathways [19][20].

Eqn. 8 expresses the rate of the chemical reaction between $^1O_2$ and HPT. The steady-state concentration of $^1O_2$ during the irradiation of a HPT aqueous solution is given by *Eqn. 9*, where I_a and Φ_Δ are the rate of absorption of photons by HPT and the quantum yield of $^1O_2$ production by HPT, respectively, and k_d is the nonradiative-deactivation rate constant in H_2O ($\tau_\Delta = 1/k_d = 3.8$ μs). k_e is not included in *Eqn. 9* because k_d is much higher than k_e [31][37]. The maximum possible value of k_r is k_t (if $k_r >> k_q$). Therefore, assuming that k_t in H_2O is equal to that obtained in D_2O (no isotope effect) and taking into account the steady-state concentration of $^1O_2$ and the initial HPT concentration, the maximum possible value for the rate of the reaction between $^1O_2$ and HPT in H_2O can be calculated ($(\mathrm{d}[\mathrm{HPT}]/\mathrm{d}t)_1 = -k_t\,[^1O_2]\,[\mathrm{HPT}]$). Under the same experimental conditions used for the determination of the quantum yields associated with the disappearance of HPT and the formation of FPT, a value of 0.66 μM/min is calculated for $(\mathrm{d}[\mathrm{HPT}]/\mathrm{d}t)_1$. This value is much lower than that obtained for the experimental rate of degradation of HPT (13.7 ± 0.8 μM/min, see above). Therefore, these results suggest that although HPT is a good $^1O_2$ sensitizer and is able to

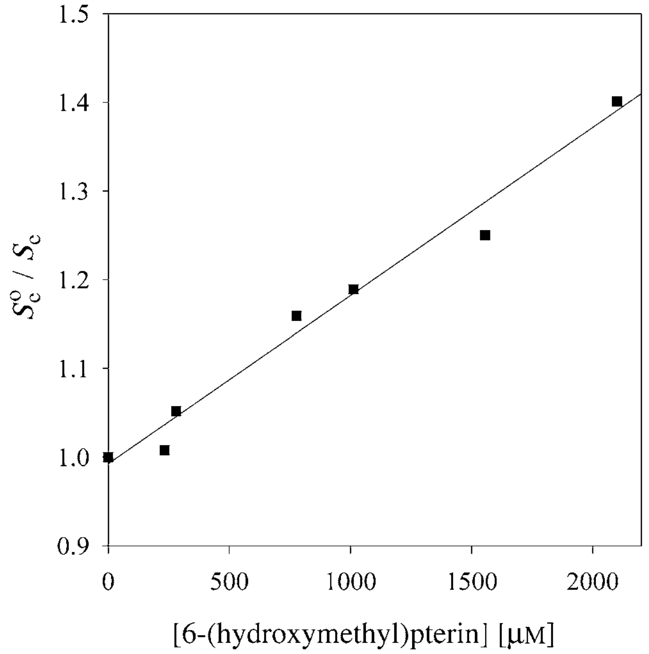


Fig. 9. Stern–Volmer *plot of the quenching of the* $^1O_2$ *near-IR luminescence by 6-(hydroxymethyl)pterin in* D_2O. Rose bengal was used as a sensitizer, λ_{ex} 547 nm.

quench $^1O_2$, the chemical reaction between both species is not quantitatively relevant as a HPT photooxidation pathway.

$$(\mathrm{d[HPT]/d}t)_1 = -k_r[^1O_2]\ [\mathrm{HPT}] \qquad (8)$$

$$[^1O_2] = \frac{I_a \Phi_\Delta}{k_d + k_t[\mathrm{HPT}]} \qquad (9)$$

Photolysis experiments were performed in D_2O to evaluate the effect of D_2O on the photooxidation pathways of HPT. Air-equilibrated solutions of HPT (260 µM) at pH 10.5 in H_2O and D_2O were irradiated. Evolutions of spectra as a function of the irradiation time were recorded for both photolysis experiments. Within experimental error, the NED spectra obtained in both photolysis experiments and the rates of absorbance changes at different wavelengths are quite similar (results not shown). These results indicate that $^1O_2$ does not participate in the main mechanism of photooxidation of HPT, being in good agreement with results presented above in this section.

Conclusions. – Photooxidation of the basic form of 6-(hydroxymethyl)pterin (HPT) shows similar patterns to those observed in the photooxidation of 6-formylpterin (FPT) [19][21] in alkaline medium. Photooxidation of HPT to FPT occurs through, at least, two steps. In the first one, after absorption of a photon and without participation of O_2, a 'red intermediate' is generated. In the second step, the intermediate reacts with O_2 yielding FPT. Due to its spectral features and reactivity, the 'red intermediate' could be 6-formyl-5,8-dihydropterin (*Scheme 2*).

During photooxidation of HPT, H_2O_2 is generated. H_2O_2 is probably a product of the thermal reaction between the 'red intermediate' and O_2. During the HPT → FPT process, the hydroxy radical ($HO^{\bullet}$) does not seem to participate actively. Generation of H_2O_2 by HPT is important from a biomedical point of view, since this activated oxygen species is involved in pathogenesis of vitiligo [43], and the accumulation of different pterin derivatives in the epidermis of the affected patients has been established.

Evidence is presented showing that HPT is a good singlet-oxygen ($^1O_2$) sensitizer under UV-A irradiation in alkaline aqueous solutions ($\Phi_{\Delta} = 0.21 \pm 0.01$). This result, in good agreement with previous studies reported on other pterin derivatives [13], suggests that the participation of pterins in biological oxidative photodynamic processes should be taken into account.

The rate constant k_t of total $^1O_2$ quenching by HPT is $3.1 \cdot 10^6$ M^{-1} s^{-1}, indicating that this compound is able to trap $^1O_2$. However, kinetic analysis and experiments in the presence of D_2O indicate that $^1O_2$ does not participate in the main pathway of the photooxidation of HPT to FPT.

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