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Study of the photolysis of folic acid and 6-formylpterin in acid aqueous solutions

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Abstract

Folic acid and 6-formylpterin solutions of pH in the range 4.5–6.0 were photolysed at 350 nm at room temperature. The photochemical reactions were followed by UV–VIS spectrophotometry, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). In the presence of oxygen, folic acid is photochemically cleavaged yielding 6-formylpterin and *p*-aminobenzoylglutamic acid. As the photolysis proceeds, 6-carboxypterin arises from 6-formylpterin, as observed when solutions only containing the latter compound are irradiated in the presence of oxygen. In the absence of oxygen, folic acid is not light-sensitive. However, when oxygen free solutions of 6-formylpterin are irradiated, a 'red intermediate' is observed, which is thermally oxidised producing 6-carboxypterin on admission of oxygen. The quantum yields of substrates disappearance and of photoproducts formation are reported.

Keywords: Folic acid; 6-Formylpterin; Photolysis

1. Introduction

Folates and pterins are heterocyclic compounds widespread in biological systems. Folic acid or pteroylglutamic acid (Fig. 1), a conjugated pterin, is a vitamin of the B group and acts as a coenzyme in reactions related to the synthesis of puric and pirimidinic bases [1]. Pterins participate in relevant biological functions [2,3], i.e. some pterin derivatives are present in butterflies [2] as natural pigments. Biopterin acts as a coenzyme in metabolic reactions involving the hydroxylation of aromatic rings.

Reports showing or suggesting that pterins are involved in different photochemical processes are frequently found in the literature. Light sensitivity of pterins derivatives is well known [4,5] and they have been found in photosensitive organs such as the eyes [6]. Moreover, it has been suggested that pterins may play some role in photosynthesis [7] and may act as blue antennas in superior plants [8] and other organisms such as the fungus *Phycomyses blakesleeanus* [9]. Also an increase of the concentration of biopterin in human skin with pigmentation disorders [10] has been observed. The folic acid derivative 5,10-methenyltetrahydrofolylpolyglutamate is present as the light-harvesting antenna in DNA photolyases, which are involved in DNA repair processes [11–13] after UV light irradiation. Ability of pterin derivatives to photosensitise DNA cleavage has been reported. In this way, photoinduced cleavage of thymus calf DNA [14] and cleavage of plasmid DNA [15] have been observed.

Despite the few reports on photochemistry [16–19] and photophysics [20–22] of pterins found in the literature, much is still left to be known on the photochemistry of folic acid and pterins. To our knowledge, only one study, analysing photochemical products of folic acid, has been reported in literature [23]. In this study, performed at pH 7, Lowry et al. [23] employed indirect methods to identify photoproducts suggesting the formation of several pteridinic derivatives. *p*-Aminobenzoylglutamic acid and 6-formylpterin were proposed as photoproducts. No information of the reaction pathways or the effect of absence of O_2, as well as quantum efficiency, were reported.

In previous communications, we reported a laser flash photolysis study on folate at pH 11 in dioxygen free solution [24] as well as the photolysis of 6-carboxypterin in alkaline and acidic aqueous solutions [19]. In the latter study, we reported the quantum efficiency of photodegradation of 6-carboxypterin and that of the formation of pterin (an observed photoproduct) and the effect of O_2 in these

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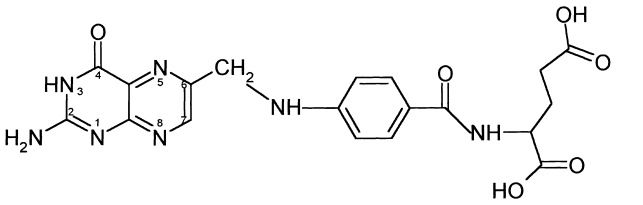


Fig. 1. Molecular structure of folic acid.

photochemical reactions. It was observed that acid and alkaline forms of 6-carboxypterin show different photochemical behaviour.

Amide group in pterins moiety suffers deprotonation to the corresponding fenolate in alkaline media. The aim of this work is to study the photolysis of the acid forms of folic acid and 6-formylpterin by UV-A light (320–400 nm). Thus, the pH conditions (5.0–6.0) were chosen, so that only the acid forms are present.

In the present work, products of photolysis of folic acid and 6-formylpterin were identified and quantified and the role of O_2 was analysed. Quantum yields were also calculated. Kinetic considerations and mechanistic aspects are discussed.

2. Experimental

Folic acid (Merck) and pterins (Schircks Laboratories) were used without further purification. Other chemicals from Sigma-Aldrich were used as received. The pH of solutions was adjusted to 4.5–6.0 by adding drops of concentrated solutions of HCl or NaOH. The ionic strength was approximately 10^{-3} M in all the experiments. pK_a values were determined from absorption changes. Measurements were performed at room temperature. The experimental absorption changes at a given wavelength can be fitted by the following equation:

$$A = 10^{-4} \times \left\{ \varepsilon_a + (\varepsilon_b - \varepsilon_a) \left[\frac{K_a}{K_a + [H^+]} \right] \right\} \quad (1)$$

Here, ε_a and ε_b are the extinction coefficients of the acid and basic forms of the species involved in the acid–base equilibrium and K_a the dissociation constant. A more detailed description of pK_a determinations can be found in the literature [25–27].

UV–VIS spectra were performed on a CARY 3 (VARIAN) spectrophotometer, using a program for smoothing and averaging signals. Measurements were made using quartz cells of 1 cm optical length and double cells of 1 cm (for irradiation) and 0.2 cm (for absorbance measurements) of optical path.

The continuous photolysis of folic acid and 6-formylpterin solutions were carried out in the presence or in the absence of air. Deaerated solutions were obtained by bubbling with oxygen-free nitrogen for 20 min. Rayonet RPR 3500 Å lamps (Southern N.E. Ultraviolet Co.) were employed.

Photolysis products were identified by analysing the absorbance change during irradiation, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

Absorption spectra of the solutions 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 normalised relative to the maximum absolute value of the absorbance difference yielding the normalised experimental difference (NED) spectrum.

NED spectra show significant differences as function of pH and O_2 concentration. However, for each particular experimental condition, NED spectra presented similar features at different irradiation times.

Reference difference (RD) and normalised reference difference (NRD) spectra were obtained from aqueous solutions of commercial standards. The RD spectra were calculated as follows: the absorption spectrum of the reactant of given experiment (folic acid or 6-formylpterin) was subtracted from the spectrum of standard solutions of the commercial compounds, assumed to be possible products of the reaction (*p*-aminobenzoylglutamic acid, pterin, xanthopterin, leucopterin, 6-formylpterin, pterin-6-carboxylic acid and 6-methylpterin). These spectra were recorded using solutions of equal concentrations for both compounds, at concentrations similar to that of the photolysis experiments. The NRD spectra were obtained by normalising RD spectra as described above.

The procedures described previously were carried for all experimental conditions reported in this paper. The comparison between NED and NRD spectra allows characterisation of the photolysis products.

TLC experiments were performed using DEAE-cellulose plates (100 μm) and aqueous NH_4Cl 0.3% (w/v) solution as eluent. Irradiated folic acid solutions (4.5×10^{-4} M) and irradiated 6-formylpterin (1.5×10^{-4} M) and aqueous solutions of pteridinic derivatives standards were developed by exposing them to 350 nm light. Under the present conditions, pterin derivatives show blue fluorescent spots, while folic acid and *p*-aminobenzoylglutamic acid do not fluoresce. In Table 1, rfs obtained for several standards solutions are reported.

Table 1
Rfs obtained for several standards solutions in TLC runs

Compound	rf
Pterin	0.49
6-Carboxypterin	0.17
6-Formylpterin	0.42
Folic acid	0.00
6-Methylpterin	0.48
6-Hydroxymethylpterin	0.53
Xanthopterin	0.38

HPLC (Konic Instruments 500 B) was employed for monitoring the reaction and identifying photoproducts, using a C18 Spherisorb S5 ODS2 (250 mm × 46 mm) column. Three different eluent were used. Eluent 1: 10% Acetronitrile:H_2O (60:40) (solution 1), 90% aqueous solution of 20 mM potassium phosphate (pH=5.5) and 2.5 mM EDTA (solution 2). Eluent 2: 7% solution 1, 93% solution 2. Eluent 3: 5% methanol, 95% aqueous solution of 50 mM sodium acetate and 5 mM citric acid (pH=5.2). A Lambda 1000 Bischoff HPLC spectrophotometer was used as detection system. For irradiated folic acid solutions, eluent 2 at 340 nm was employed for monitoring pterin derivatives and folic acid, whereas eluent 3 at 280 nm was employed for identification and quantification of *p*-aminobenzoylglutamic acid, which does not absorb at 340 nm. For irradiated 6-formylpterin solutions, eluent 1 at 280 nm was used.

The quantum yields of folic acid and 6-formylpterin disappearance and 6-formylpterin and 6-carboxypterin formation were determined in experiments performed under different conditions. A chemical actinometer, ($[Co^{III}(NH_3)_5Br]^{2+}$) was employed for the measurements of photon rates [28]. When possible, the initial concentration of reactants 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. Aqueous solutions of commercial standards were employed for obtaining the corresponding calibration curves.

The oxygen 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 [29].

Photolysis, in the presence of furfuryl alcohol (10 mM) (Riedel-de Haën) were carried out. This compound has a high reactivity with singlet oxygen [30] and because of that greatly reduces its concentration during the photolysis. In this group of experiments, folic acid, 6-formylpterin and 6-carboxypterin concentrations were determined as a function of irradiation time by HPLC measurements. Results are compared with those obtained in the absence of the selective singlet oxygen scavenger.

3. Results and discussion

3.1. pK_as study

Folic acid and pterins exhibit several dissociation equilibrium's. As reported by Albert [25] for several pterin derivatives, the amide group is relevant only in the present pH conditions, as the pK_as of other functional groups of pterin moiety (i.e. 2-amino group) are lower than two and carboxylic groups lower than 4.5. The pK_as of amide groups of folic acid and 6-carboxypterin were determined previously [27] and are 8.10 and 7.88, respectively. To our knowledge, the pK_a values of the amide group in 6-formylpterin and the 6-carboxylic group in 6-carboxypterin are not reported in literature. We have, therefore, determined the later pK_a values (7.33 and 3.05, respectively).

3.2. Photolysis of folic acid in the presence of O_2

Important changes in the absorption spectra of folic acid solutions were observed after irradiation, as shown in Fig. 2. The NED spectrum in this case is similar to the NRD spectrum obtained by subtracting the spectrum of a standard solution of folic acid from the spectrum of a standard solution of 6-formylpterin and *p*-aminobenzoylglutamic acid. The NED and NRD spectra were obtained as described in the experimental section. The agreement observed between the two spectrum (Fig. 2b) suggests that, under these irradiation conditions, the folic acid molecule undergoes cleavage and oxidation yielding 6-formylpterin and *p*-aminobenzoylglutamic acid.

TLC experiments show the presence of two fluorescent components in the irradiated solutions. The rfs observed correspond to 6-carboxypterin and 6-formylpterin used as standards. In experiments performed at different irradiation times, it can be noticed that 6-carboxypterin appears in a later stage than 6-formylpterin.

Presence of 6-formylpterin and 6-carboxypterin in irradiated solutions of folic acid are confirmed by HPLC

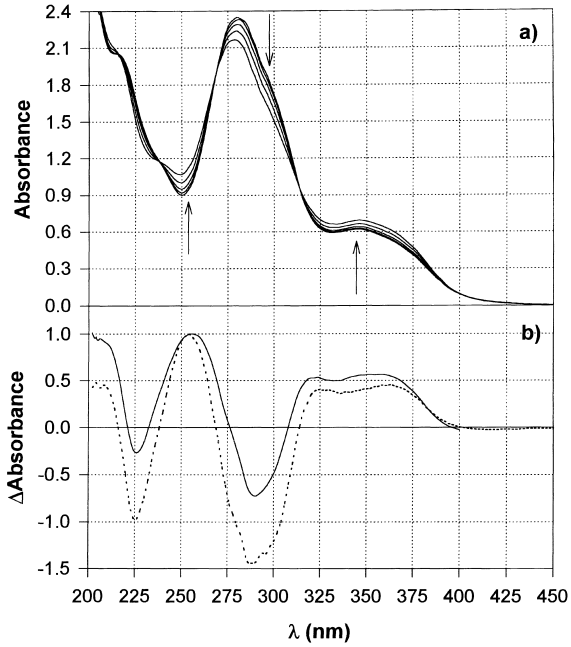


Fig. 2. (a) Evolution of the absorption spectra of irradiated solutions of folic acid (450 μM at pH 6.0), as a function of time. Experiment performed in the presence of O_2. Spectra were recorded every 2 min, arrows indicate the changes observed at different wavelengths. (b) (——) NED spectrum obtained by subtracting the initial folic acid spectrum from the corresponding spectrum after 8 min of photolysis. (-----) NRD spectrum obtained by subtracting the spectrum of a standard solution of folic acid from the spectrum of a standard solution of 6-formylpterin and *p*-aminobenzoylglutamic acid, at the same concentration and pH.

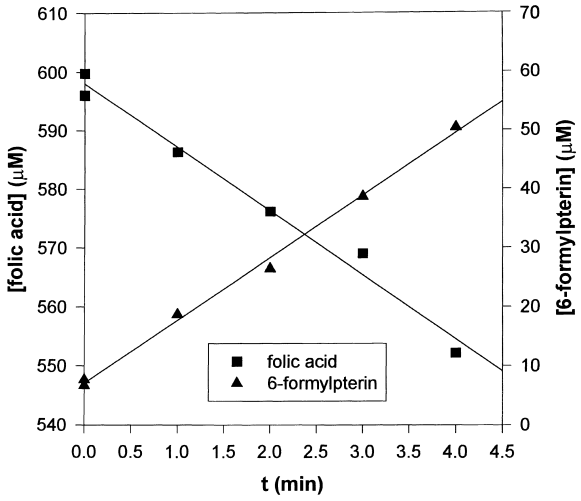


Fig. 3. Evolution of folic acid and 6-formylpterin concentrations in irradiated solution of folic acid 600 μM (pH=6.0) as a function of time. HPLC analysis of first 5 min of photolysis.

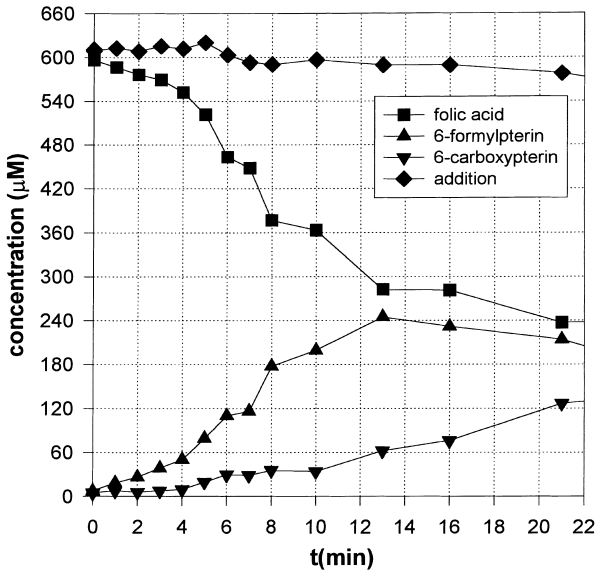


Fig. 4. Evolution of folic acid, 6-formylpterin and 6-carboxypterin concentrations in irradiated solution of folic acid 600 μM (pH=6.0) as a function of time. HPLC analysis of 22 min of photolysis. (◆) Algebraic addition of folic acid, 6-formylpterin and 6-carboxypterin concentrations, for each time.

experiments performed using eluent 2. The presence of *p*-aminobenzoylglutamic acid is detected employing eluent 3.

Folic acid, 6-formylpterin and 6-carboxypterin 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 folic acid (>500 μM) were irradiated. Second group was performed on folic acid and 6-formylpterin mixtures.

The first group of experiments show that, in the first 4 min of irradiation, the evolutions of the concentrations of folic acid and 6-formylpterin follow a zero order rate law, as shown in Fig. 3. In this first stage, no significant increase of 6-carboxypterin concentration is observed. Under these concentration conditions, it can be assumed that, in this time range, the whole light is absorbed by folic acid. The quantum yield associated with the disappearance of folic acid was measured and a value of 2.5×10^{-2} was obtained. On the other hand, a value of 2.5×10^{-2} was also obtained for the quantum yield of 6-formylpterin formation. Therefore, consumption of folic acid to lead 6-formylpterin is the only process that take place in the analysed time range.

After 4 min of irradiation, important increases on the rate of photodegradation of folic acid and on the rate of formation of 6-formylpterin are observed, as shown in Fig. 4. This facts coincide temporally with an increase of 6-carboxypterin concentration. As it will be shown below, 6-carboxypterin is a photoproduct generated from 6-formylpterin. Therefore, beginning from approximately 5 min, some significant proportion of light is absorbed by 6-formylpterin. Thus, the chemical transformation from folic acid to 6-formylpterin seems to be enhanced trough participation of excited 6-formylpterin.

As can be seen in Fig. 4, during the first 25 min of irradiation, under these conditions, the total concentration of pterins in μM are almost constant, thus indicating that 6-formylpterin and 6-carboxypterin are the main photoproducts. In a later stage of the photolysis, the 6-carboxypterin suffers photodegradation itself as reported in the literature [19]. Therefore, the slightly decrease of total concentration of pterins, observed after 30–40 min of irradiation (results not shown), can be mainly associated to the photodegradation of 6-carboxypterin.

The second group of experiments were performed in such a way that only a low proportion of the incident light is absorbed by folic acid, whereas a higher proportion is absorbed by 6-formylpterin. In the experiment shown in Fig. 5,

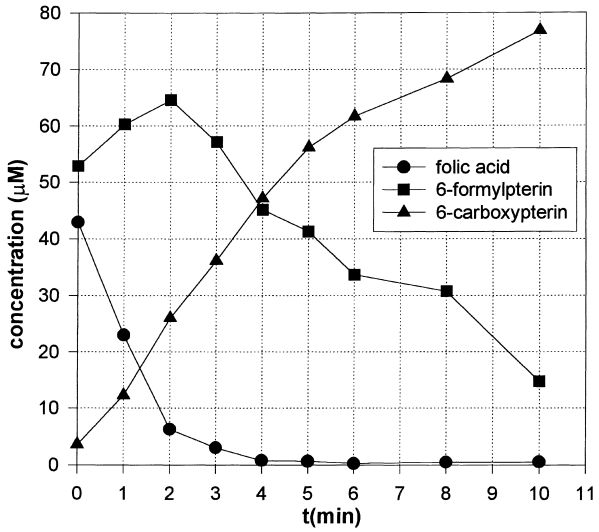


Fig. 5. Evolution of folic acid, 6-formylpterin and 6-carboxypterin concentrations in irradiated mixture solution of folic acid (43 μM) and 6-formylpterin (53 μM) (pH=5.8) as a function of time. HPLC analysis of 10 min of photolysis.

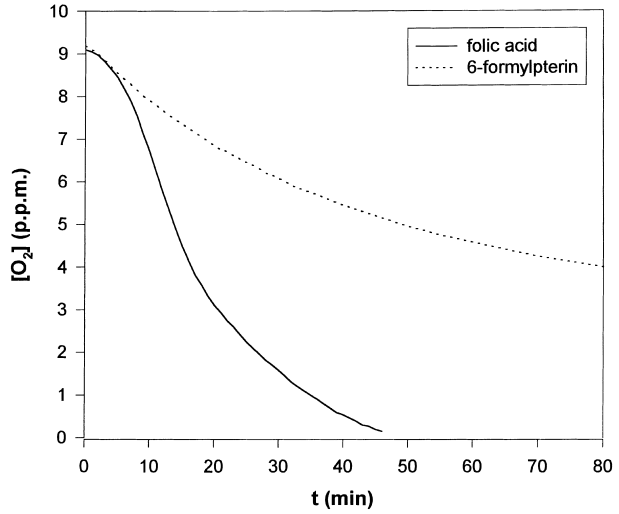


Fig. 6. Time evolution of the O_2 concentration in irradiated solutions of folic acid (156 μM) and 6-formylpterin (200 μM).

a mixture solution of folic acid (43 μM) and 6-formylpterin (53 μM) was irradiated. An initial rate of consumption of 2.76 μM/min is estimated, under these conditions, taking the intensity absorbed by folic acid as 25% of the incident light and the quantum yield previously calculated (2.5×10^{-2}). However, a rate of 20.0 μM/min was obtained for the first minute of photolysis. These results confirm that absorption of light by 6-formylpterin induces the conversion of folic acid to 6-formylpterin.

In another set of experiments, a significant decrease on O_2 concentration, during the photolysis was observed, indicating that photooxidation of folic acid is taking place. As shown in Fig. 6, after a few minutes of irradiation, an increase on the rate of consumption of O_2 is observed. The degradation of folic acid induced by 6-formylpterin and the photooxidation of 6-formylpterin may explain this observation.

3.3. Photolysis of folic acid in the absence of O_2

Completely different results are obtained in experiments performed in nitrogen saturated solutions. No significant changes in the absorption spectra of folic acid solutions (450 μM and pH=5.8) were observed during 50 min of irradiation. Moreover, TLC experiments carried out on irradiated solutions show that no fluorescent products are detected.

HPLC measurements performed on an irradiated solution of folic acid (600 μM and pH=5.8) show that folic acid concentration does not decrease after 30 min of irradiation. Accordingly, no new substrates were detected. Therefore, folic acid does not undergo any chemical modification under irradiation at 350 nm in the absence of O_2.

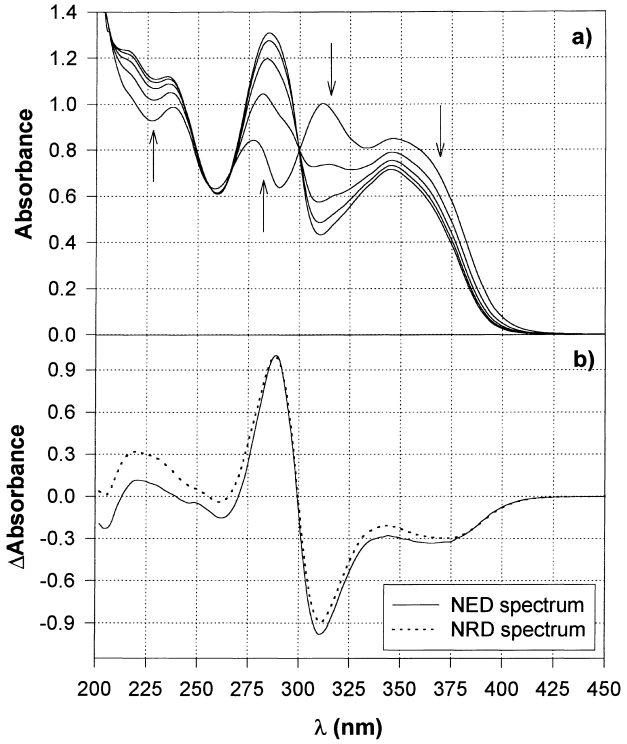


Fig. 7. (a) Evolution of the absorption spectra of irradiated solutions of 6-formylpterin (112 μM at pH 6), as a function of time. Experiment performed in the presence of O_2. Spectra were recorded every 2 min, arrows indicate the changes observed at different wavelengths. (b) (——) NED spectrum obtained by subtracting the initial 6-formylpterin spectrum from the corresponding spectrum after 8 min of photolysis. (-----) NRD spectrum obtained by subtracting the spectrum of 6-formylpterin and 6-carboxypterin at the same concentration and pH.

3.4. Photolysis of 6-formylpterin in the presence of O_2

The spectrum of 6-formylpterin solutions changes steadily with the irradiation time as shown in Fig. 7. As can be seen in Fig. 7b, the NED spectrum is similar to the NRD spectrum obtained by subtracting the spectrum of a standard solution of 6-formylpterin from the spectrum of a standard solution of 6-carboxypterin.

As observed for irradiated solutions of folic acid, TLC experiments show the presence of two fluorescent components in the irradiated solutions of 6-formylpterin. The rfs correspond to 6-carboxypterin and 6-formylpterin used as standard.

Moreover, HPLC measurements show that 6-carboxypterin arises from 6-formylpterin photolysis. No other photoproducts were detected in the first minutes of photolysis. Evolution of concentrations, as a function of time, can be appreciated in Fig. 8. The measured quantum yields associated with the disappearance of 6-formylpterin and with formation of 6-carboxypterin are 4.0×10^{-2} and 3.7×10^{-2}, respectively. As shown in Fig. 6, O_2 consumption is observed during photolysis. These results show that 6-formylpterin suffers photooxidation on 6-formyl group to lead corresponding 6-carboxy derivative.

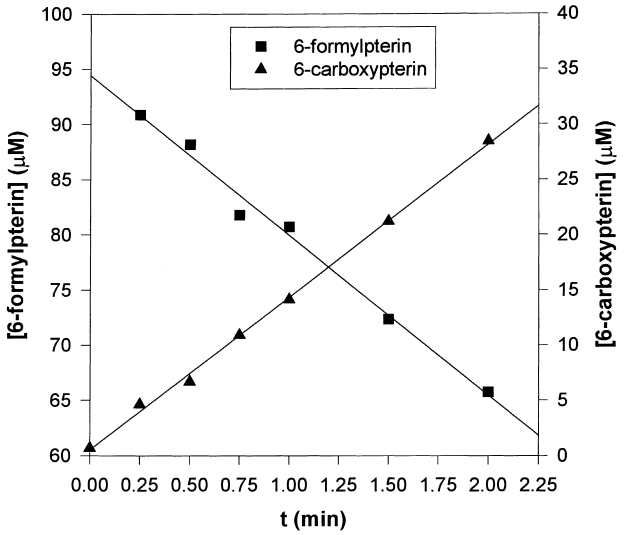


Fig. 8. Evolution of 6-formylpterin and 6-carboxypterin concentrations in irradiated solution of 6-formylpterin (95 μM, pH=6.0) as a function of time. HPLC analysis of first 2 min of photolysis.

3.5. Photolysis of 6-formylpterin in the absence of O_2

Solutions of 6-formylpterin present a yellowish colour. When a deaerated solution of 6-formylpterin is irradiated with UV-A it becomes orange-red in a few minutes. As can be appreciated in Fig. 9, photolysis of 6-formylpterin, under anaerobic conditions, leads to the formation of a 'red compound' with a longwave absorbing band of low extinction at 480 nm. Commercial available pteridinic derivative standards, not present such spectrophotometrical characteristics and, therefore, NED spectrum of this photolysis does not agree with NRD spectra obtained from commercial standards solutions.

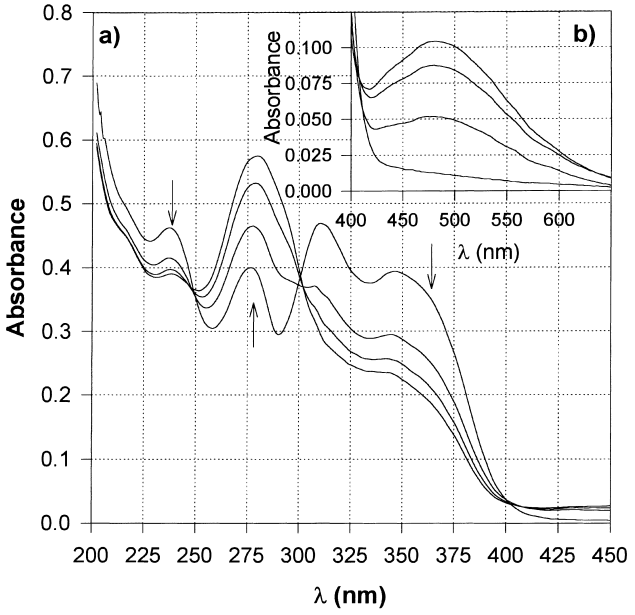


Fig. 9. Evolution of the absorption spectra of irradiated solutions of 6-formylpterin (200 μM at pH 5.8), as a function of time. Experiment performed in the absence of O_2. Spectra were recorded every 2 min, arrows indicate the changes observed at different wavelengths. (a) 0.2 cm optical length; (b) 1.0 cm optical length.

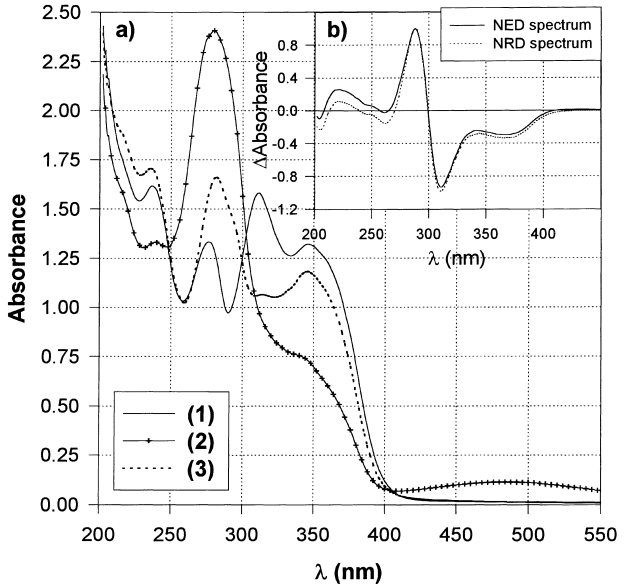


Fig. 10. (a) Spectra of 6-formylpterin solutions (134 μM, pH=5.0). (1) Spectrum of non-irradiated solution; (2) Spectrum of 10 min irradiated solution, under anaerobic conditions; (3) Spectrum of 10 min irradiated solution, under anaerobic conditions, and then aerated. (b) (——) NED spectrum obtained by subtracting the initial spectrum (1) from the corresponding spectrum (3). (-----) NRD spectrum obtained by subtracting the spectrum of 6-formylpterin and 6-carboxypterin at the same concentration and pH.

Spectral changes take place after aeration of irradiated 6-formylpterin solution in the absence of O_2, as shown in Fig. 10. NED spectrum, obtained from subtracting the spectrum of non-irradiated solution from that of an irradiated and then aerated solution, is in agreement with corresponding NRD spectrum, obtained by subtracting the spectrum of 6-formylpterin and 6-carboxypterin standard solutions (Fig. 10b). These results suggest that this 'red compound' suffer spontaneous oxidation to 6-carboxypterin on admission of oxygen.

TLC runs of the previous experiments are very similar to those observed for experiments performed in the presence of O_2, indeed the corresponding 6-carboxypterin spot can be observed. In the same way, HPLC experiments show the decrease of 6-formylpterin concentration and increase of 6-carboxypterin concentration (results not shown). The quantum yield, associated with the disappearance of 6-formylpterin, was measured and a value of 4.7×10^{-2} was obtained.

From HPLC measurements, non-photolised concentration of 6-formylpterin was obtained for different irradiation times. Then corresponding spectra were obtained. Subtracting these spectra from the spectra of irradiated solutions,

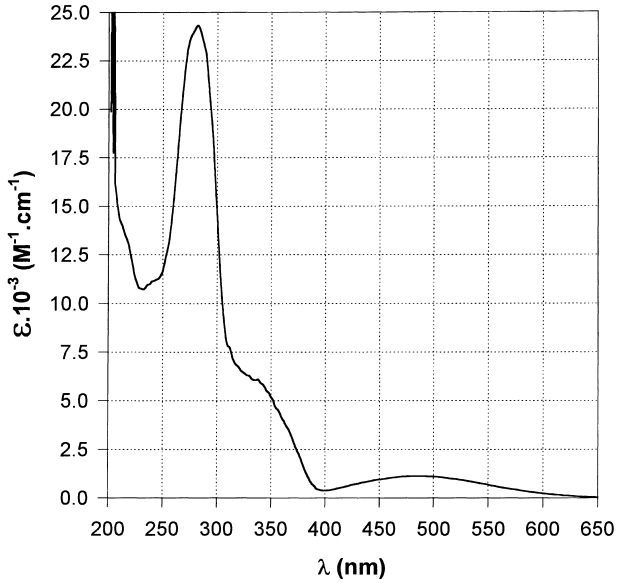


Fig. 11. Spectrum of intermediate detected on irradiated, under anaerobic conditions, 6-formylpterin solutions.

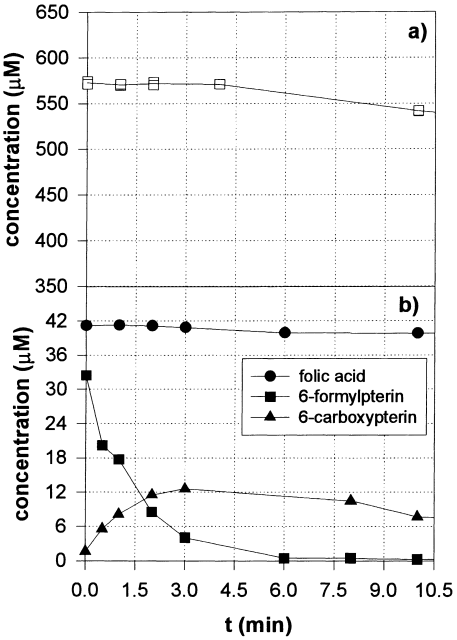


Fig. 12. (a) Time evolution of folic acid concentration in irradiated solution of folic acid 570 μM (pH=6.0) in the presence of furfuryl alcohol (10 mM); (b) Time evolution of folic acid, 6-formylpterin and 6-carboxypterin concentrations in irradiated mixture solution of folic acid (41 μM) and 6-formylpterin (33 μM) (pH=6.0) in the presence of furfuryl alcohol (10 mM).

under anaerobic conditions, the spectra of the 'red compound' was obtained, assuming that conversion to 'red compound' is the only pathway of 6-formylpterin consumption. This spectrum is shown in Fig. 11.

3.6. Photolysis of folic acid and 6-formylpterin in the presence of singlet oxygen scavenger

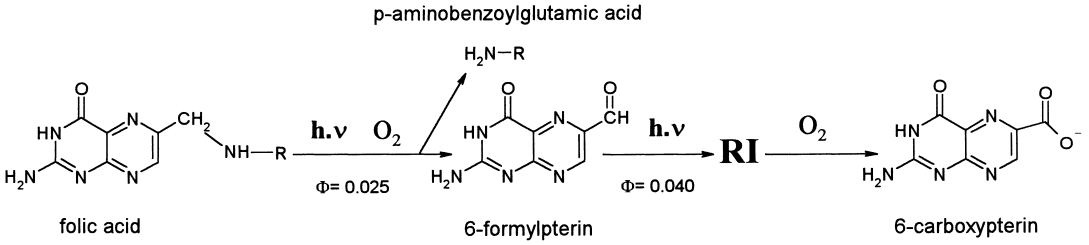
Pterins derivatives are able to produce singlet oxygen [31,32]. Photolysis, in the presence of furfuryl alcohol, a selective scavenger, were carried out to investigate if singlet oxygen are involved in the mechanism of photodegradations of folic acid and 6-formylpterin.

As shown in Fig. 12a, folic acid photolysis proceeds at a comparatively very slow rate under these conditions. In the experiment shown in Fig. 12b, a starting solution containing folic acid (41 μM) and 6-formylpterin (33 μM) was irradiated. It can be appreciated that folic acid does not suffer significant degradation. As observed in these experiments, evolution of folic acid concentration differs strongly from those corresponding to the analogous experiments, which were carried out in the absence of furfuryl alcohol (Figs. 3–5). These results suggest that singlet oxygen is involved in direct photolysis of folic acid and that 6-formylpterin generates singlet oxygen accelerating the decomposition of folic acid.

On the other hand, photooxidation of 6-formylpterin to yield 6-carboxypterin is not inhibited by furfuryl alcohol (Fig. 12b) suggesting that singlet oxygen seems to be not involved in this reaction.

4. Conclusions

Scheme 1 summarises the results obtained. In the absence of oxygen, folic acid is photostable, whereas excitation in the presence of oxygen leads to cleavage and oxidation of the molecule, yielding 6-formylpterin and *p*-aminobenzoylglutamic acid as photoproducts. Singlet oxygen,



Scheme 1.

probably produced by electronically excited states of folic acid molecule, is the oxidant agent which start the degradation reaction. No other photoproducts were detected under our experimental conditions.

This degradation reaction of folic acid is accelerated in the presence of 6-formylpterin. This process requires photoexcitation of 6-formylpterin, not requires the absorption of light by folic acid and is inhibited by singlet oxygen scavenger. Therefore, excitation of 6-formylpterin leads to the generation of singlet oxygen that induces the degradation of folic acid.

To propose a detailed mechanism of folic acid photodegradation, further research is in the process in our laboratory. In another direction, further investigations, to get a complete view of the ability of the different pterins for yielding singlet oxygen in aqueous solutions, are under progress.

Photooxidation of 6-formylpterin to 6-carboxypterin occurs through, at least, two steps. In the first one, the excitation of the molecule leads to the formation of a 'red intermediate'. In the second step, the intermediate reacts with oxygen yielding 6-carboxypterin. Singlet oxygen seems to be not involved in this reaction.

As it was proposed in literature [16,17], the long wavelength absorption at 480–500 nm of low intensity seems to be characteristic for 6-acyl-5,8-dihydropterins. These compounds suffer spontaneous dehydrogenation to corresponding 6-acylpterin on admission of oxygen. Due to its spectral features and reactivity, the 'red intermediate' detected in our experiments could be 6-carboxy-5,8-dihydropterin. Further investigations to obtain more evidence in this way are under progress.

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