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Reaction between 7,8-dihydropterins and hydrogen peroxide under physiological conditions

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ABSTRACT

In vitiligo, a common skin disorder that produces white patches of depigmentation, 7,8-dihydropterins accumulate in the presence of high concentration of H_2O_2. In this work, we present a study of the reaction between 7,8-dihydropterins and H_2O_2. The rate of the reaction, as well as the products formed, strongly depend on the chemical structure of the substituents. Electron-donor groups as substituents are the most reactive derivatives and undergo oxidation of the pterin moiety. The corresponding bimolecular rate constants at 37 °C in neutral aqueous solutions are reported. The biological implications of the results obtained are also discussed.

1. Introduction

Pterins, heterocyclic compounds widespread in biological systems, are derived from 2-aminopteridin-4-(3*H*)-one or pterin. The most common pterin derivatives are 6-substituted compounds. Pterins can exist in different oxidation states and be divided into two classes according to this property: (a) oxidized or aromatic pterins containing the pyrazino[2,3-*d*]pyrimidine ring structure and (b) reduced pterins (Fig. 1). Within the latter group, 7,8-dihydropterins and 5,6,7,8-tetrahydropterins (denoted throughout as dihydropterins and tetrahydropterins, respectively) are the most important derivatives due to their biological activity, e.g., dihydroneopterin is secreted during the oxidative burst of stimulated macrophages,[1] dihydrobiopterin and tetrahydrobiopterin are involved in the metabolism of aminoacids,[2] and reduced derivatives of folic acid participate in methionine and nucleotide biosynthesis.[3] The chemistry of dihydropterins has been studied in detail.[4]

Depending on their oxidation state, pterins have totally different reactivities toward oxidizing agents, e.g., whereas dihydropterins are very efficient singlet oxygen ($^1O_2$) quenchers,[5] the reactivity of oxidized pterins toward $^1O_2$ is relatively low.[6] In air-equilibrated aqueous solutions, tetrahydropterins are rapidly oxidized by dissolved molecular oxygen in its ground state (O_2), whereas dihydropterins are relatively stable.[7–9] The oxidation of these compounds is of biological interest because they, under several pathological situations, are generated and accumulated in oxidative environments. For instance, in the skin of patients suffering vitiligo,[10] a common disorder that produces white patches of depigmentation, several reduced and oxidized pterins accumulate in the affected areas.[11,12] Hydrogen peroxide (H_2O_2) participates in the pathogenesis of this disease and is also accumulated in the depigmented epidermis.[12,13]

The oxidation of dihydrobiopterin by H_2O_2 to yield biopterin has been suggested,[14] but, despite the potential implications of this reaction, it has not been studied in detail. In contrast, the reaction between dihydroneopterin and H_2O_2 was investigated in more depth and dihydroxantopterin was reported as the main oxidation product.[15] However, to the best of our knowledge, the kinetics of these reactions and the oxidation of other dihydropterin derivatives with H_2O_2 have not been investigated at all.

In the context of our investigation on reactions between dihydropterins and different oxidizing agents, in this article we describe the oxidation of a group of 6-substituted dihydropterins by H_2O_2 in aqueous solutions, under physiological conditions (pH=7.1±0.1; T=37 °C). We investigated derivatives with substituents of different molecular weights and with different functional groups: 6-methyldihydropterin, 6,7-dimethyldihydropterin, dihydrobiopterin, dihydroneopterin, 6-formyldihydropterin, dihydroxanthopterin, and dihydrofolic acid (Fig. 1). The reaction products, in most cases, were identified. Kinetics of the reactions was analyzed and the

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R	Dihydroderivative	Oxidized pterin
-$(CHOH)_2$-CH_2OH	dihydroneopterin (DHNPT)	neopterin (NPT)
-$(CHOH)_2$-CH_3	dihydrobiopterin (DHBPT)	biopterin (BPT)
-CH_3	6-methyldihydropterin (MDHPT)	6-methylpterin (MPT)
-CH_3	6,7-dimethyldihydropterin (DMDHPT)	6,7-dimethylpterin (DMPT)
(*)	dihydrofolic acid (DHFA)	folic acid (FA)
-CHO	6-formyldihydropterin (FDHPT)	6-formylpterin (FPT)

PABA-Glu dihydroxanthopterin (DHXPT) xanthopterin (XPT)

Figure 1. Molecular structures of the dihydropterins investigated at physiological pH (neutral forms); structures of corresponding oxidized pterins are also represented. DMDHPT and DMPT have an additional methyl group at position 7 of the pterin moiety.

corresponding bimolecular rate constants were determined. We discuss the effects of the structural features of the substituents and the biological implications of the results obtained.

2. Results and discussion

The chemical reactions between hydrogen peroxide (H_2O_2) and dihydropterins in air-equilibrated aqueous solution (pH=7.0–7.2) were investigated by UV–vis spectrophotometry and HPLC analysis at 37 °C. The chemical changes after mixing the reactants were followed as a function of time. Dihydropterins slowly react with dissolved O_2 and the corresponding half life values ($t_{1/2}$) in air-equilibrated aqueous solution have been determined.[9] Taking into account these values, the rate of the oxidation of dihydropterins by O_2 was calculated for the initial conditions of each experiment. In almost all experiments, the rate of the consumption of reactants by O_2 was negligible in comparison with the rate of the reaction with H_2O_2. In a very few experiments performed at low H_2O_2 concentration, the oxidation of dihydropterin derivatives by O_2 was significant. In these cases, this reaction was taken into account and the corresponding corrections were made in kinetic analysis (vide infra).

2.1. Oxidation of dihydrobiopterin, dihydroneopterin, and 6-formyldihydropterin

Significant spectral changes were observed in aqueous solutions of dihydrobiopterin (DHBPT), dihydroneopterin (DHNPT), and 6-formyldihydropterin (FDHPT) after adding H_2O_2 aqueous solution at 37 °C (Figs. 2a, 3a, and 4a). In all cases, blue shifts were observed for the corresponding low-energy bands. Spectral analysis suggested the presence of dihydroxanthopterin (DHXPT) as the main product of the three oxidation reactions: the normalized-experimental-difference (NED) spectra were similar to the corresponding normalized-reference-difference (NRD) spectra, obtained from standard solutions of the reactants and DHXPT (see Section 4).

The formation of DHXPT was confirmed by means of HPLC analysis. The concentrations of reactants (DHBPT, DHNPT and FDHPT) and DHXPT were determined as a function of time (Figs. 2a, 3a, and 4a). For all compounds, the fraction of consumed reactant converted into DHXPT was more than 90%. Therefore, cleavage of the substituent and oxidation of C6 constitute the main pathway of the reaction of DHNPT, DHBPT, and FDHPT with H_2O_2 (Scheme 1). These results are in agreement with previous studies that reported DHXPT as a typical oxidation product of dihydropterin derivatives bearing a –CHOH– group linked to C6.[4,16]

It is interesting to compare these results to those obtained in previous studies on oxidation of dihydropterins. In particular, oxidation by H_2O_2 leads to the same product (DHXPT) as that found in the oxidation by dissolved O_2,[9] although this reaction is much slower (vide infra). In contrast, the reaction of the studied dihydropterins with $^1O_2$ is very fast and leads to the oxidation of the pterin moiety, yielding the analogous oxidized pterin (biopterin (BPT), neopterin (NPT), 6-formylpterin (FPT), Fig. 1), and non-pterinic products, where the pterin moiety was oxidized and cleaved.[5]

It is also worth mentioning the biological implications of these results, especially those from the reaction of DHBPT. Both DHBPT and H_2O_2 accumulate in the skin of patients affected by vitiligo.[11,12] Therefore, the oxidation of DHBPT and the consequent generation of DHXPT in vivo via the reaction described in this section should be taken into account.

2.2. Oxidation of dihydrofolic acid

The reaction between dihydrofolic acid (DHFA) and H_2O_2 (Fig. 5) leads to the formation of several products. HPLC analysis revealed that the main product is DHXPT (~60%) (Scheme 2, pathway a). In addition, folic acid (FA) (~40%) and a very small amount of FDHPT (<1%) were also detected (Scheme 2, pathways b and c). Oxidation of DHFA is important from a biological point of view since folic acid and its reduced forms are the most abundant pterin derivatives in living systems.

In this case, there seems to be at least two parallel pathways: the oxidation of the pyrazine ring to yield the corresponding oxidized pterin derivative (FA) and the break of the substituent by oxidation at the C-6 or C-9 position to yield DHXPT or FDHPT, respectively. The former pathway was already reported for the oxidation of

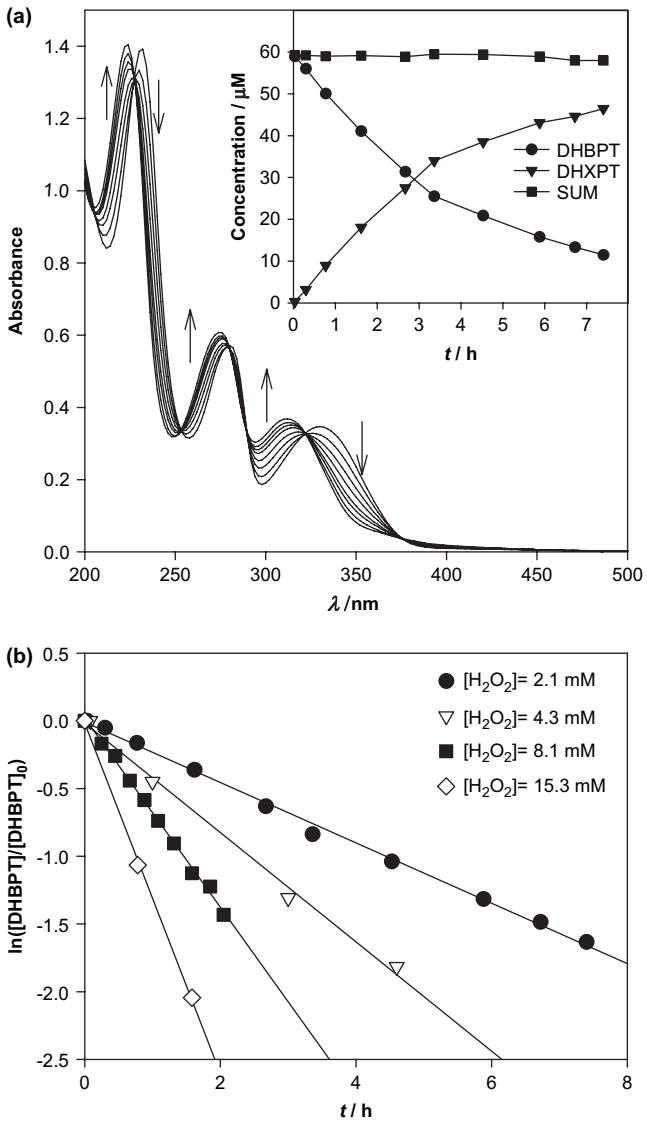


Figure 2. Reaction between DHBPT and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of product (DHXPT). Initial concentrations: $[DHBPT]_0$= 60 μM, $[H_2O_2]_0$=2.1 mM. (b) First-order plots of the oxidation of DHBPT by H_2O_2 at different concentrations (Eq. 4, Section 4). Concentrations determined by HPLC analysis.

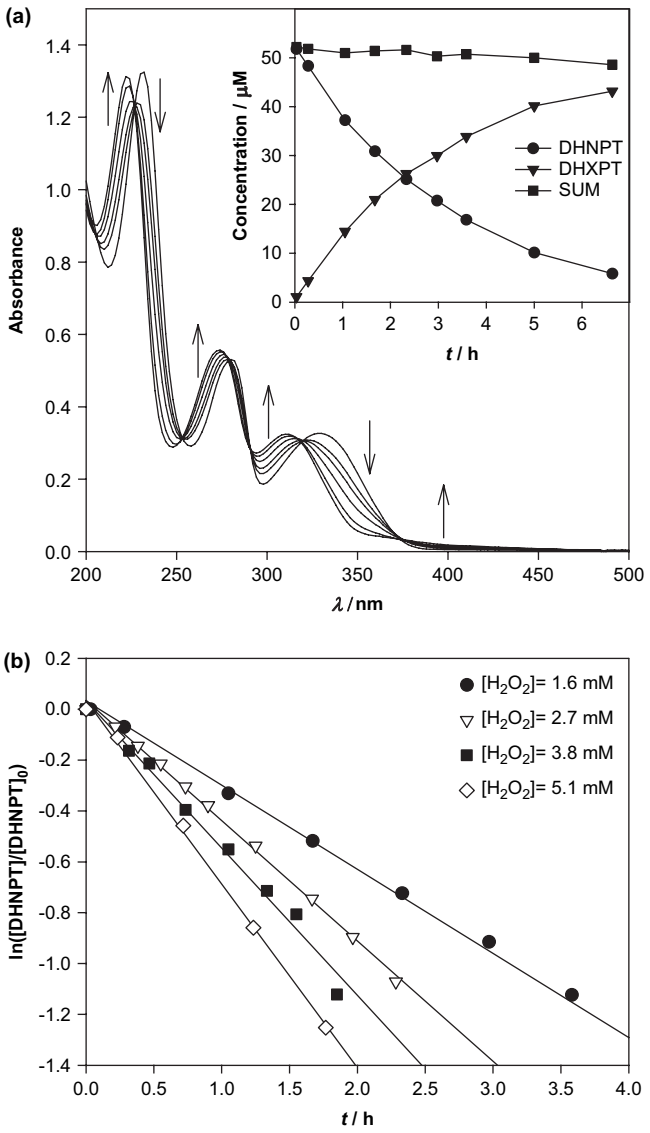


Figure 3. Reaction between DHNPT and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of product (DHXPT). Initial concentrations: $[DHNPT]_0$= 51 μM, $[H_2O_2]_0$=1.6 mM. (b) First-order plots of the oxidation of DHNPT by H_2O_2 at different concentrations (Eq. 4, Section 4). Concentrations determined by HPLC analysis.

DHFA by $^1O_2$,[5] whereas the latter one was reported for the oxidation of DHFA by dissolved O_2.[9]

2.3. Oxidation of dihydroxanthopterin

Since DHXPT is the main product of the oxidation of the most biologically important dihydropterins (DHNPT, DHBPT, and DHFA) by H_2O_2, the reaction between this reactive oxygen species and DHXPT in aqueous solution was also investigated. The oxidation is very slow compared to those presented in the previous paragraphs; e.g., after mixing DHXPT with H_2O_2 a slow decay of the typical absorption band of DHXPT with a maximum at 310 nm and a very slight increase in the absorption in the region 360–420 nm were observed (Fig. 6a).

Xanthopterin (XPT) (Fig. 1), in very low concentration, was detected by HPLC analysis. The corresponding concentration profiles (Fig. 6a) showed that, as expected, the consumption of DHXPT is relatively slow (vide infra) and its conversion into the corresponding oxidized form is very small (<5%). In addition to XPT, three other products were detected by HPLC analysis, all of them having short retention times. Taking into account the experimental conditions of our chromatographic analysis (see Section 4), this fact suggests that such additional products are very polar substances. The corresponding absorption spectra were recorded using the DAD detector of the HPLC equipment. Such analyses, carried out especially for long times (t>24 hs) where conversion of the reactant was high, suggested that the pterin moiety was oxidized and cleaved, yielding a group of non-pterinic products, i.e., the characteristic absorption bands of oxidized pterins and dihydropterins in the UV-A region were lost.

2.4. Oxidation of 6-methyldihydropterin and 6,7-dimethyldihydropterin

Relatively fast spectral changes were observed when aqueous solutions of 6-methyldihydropterin (MDHPT) and 6,7-

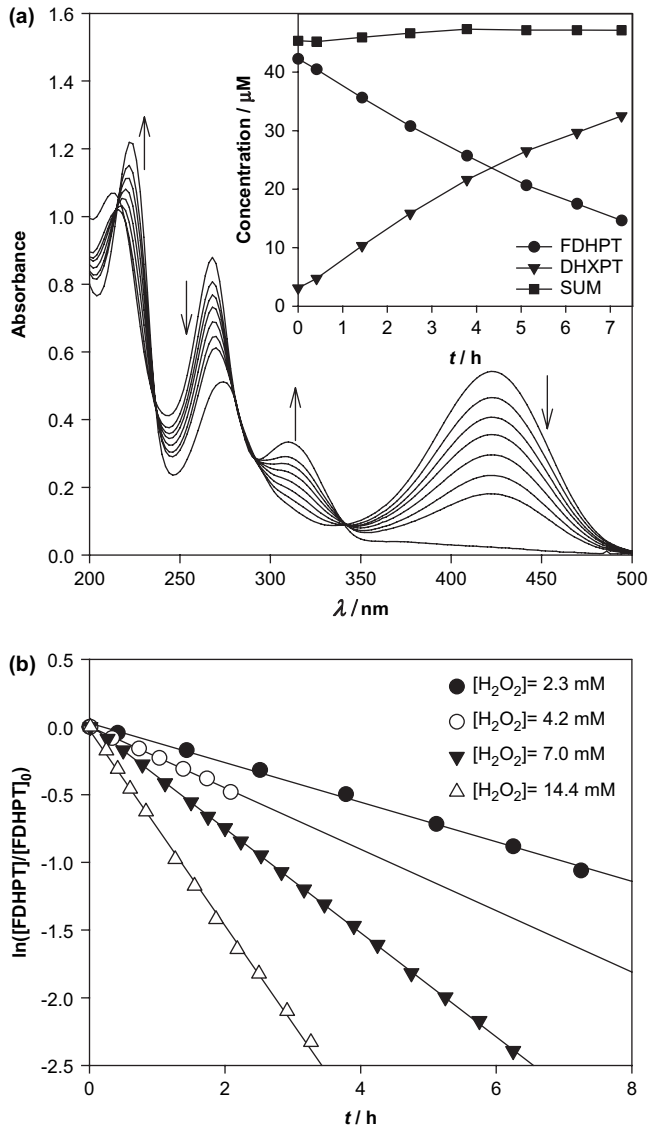


Figure 4. Reaction between FDHPT and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of product (DHXPT). Initial concentrations: $[FDHPT]_0$=42 μM, $[H_2O_2]_0$=2.3 mM. (b) First-order plots of the oxidation of FDHPT by H_2O_2 at different concentrations (Eq. 4, Section 4). DHXPT concentration determined by HPLC, FDHPT concentration determined by HPLC and spectrophotometric analyses.

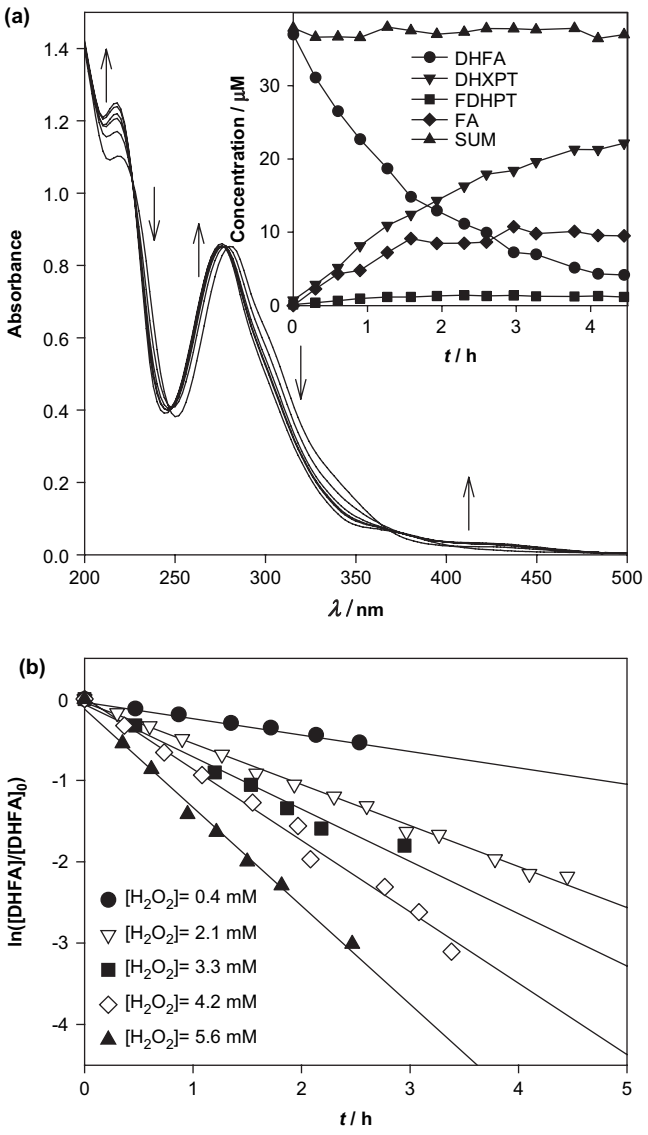
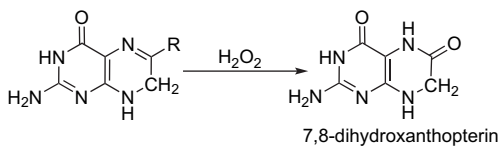


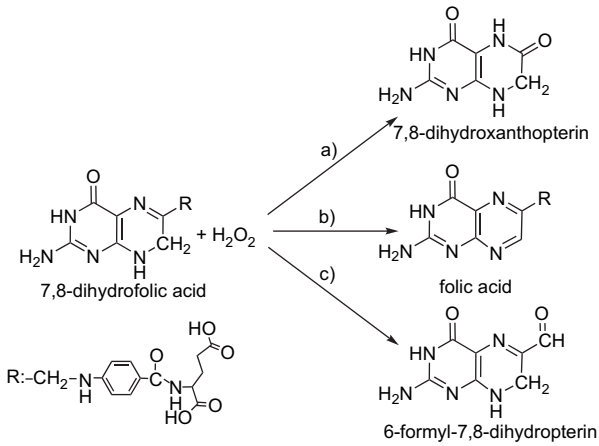
Figure 5. Reaction between DHFA and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of products (DHXPT, FA and FDHPT). Initial concentrations: $[DHFA]_0$=40 μM, $[H_2O_2]_0$=2.1 mM. (b) First-order plots of the oxidation of DHFA by H_2O_2 at different concentrations (Eq. 4, Section 4). DHXPT, FA, and FDHPT concentrations determined by HPLC and spectrophotometric analyses.

dimethyldihydropterin (DMDHPT) were mixed with aqueous H_2O_2 at 37 °C (Figs. 7a and 8a). The evolution of the spectra showed an absorbance increase at λ>340 nm, where oxidized pterins show a typical band.[17] On the other hand, no absorbance increase was observed at $\lambda\sim$310 nm. This behavior suggests that DHXPT is not the main product of the oxidation of MDHPT and DMDHPT by H_2O_2.

Moreover, HPLC analysis revealed that DHXPT is not present in the reaction mixture and that 6-methylpterin (MPT) and 6,7-dimethylpterin (DMPT) (Fig. 1) are products of the reactions of H_2O_2



Scheme 1. Oxidations of DHNPT, DHBPT, and FDHPT by H_2O_2 in neutral aqueous solution at 37 °C.



Scheme 2. Reaction between DHFA and H_2O_2 in neutral aqueous solution at 37 °C.

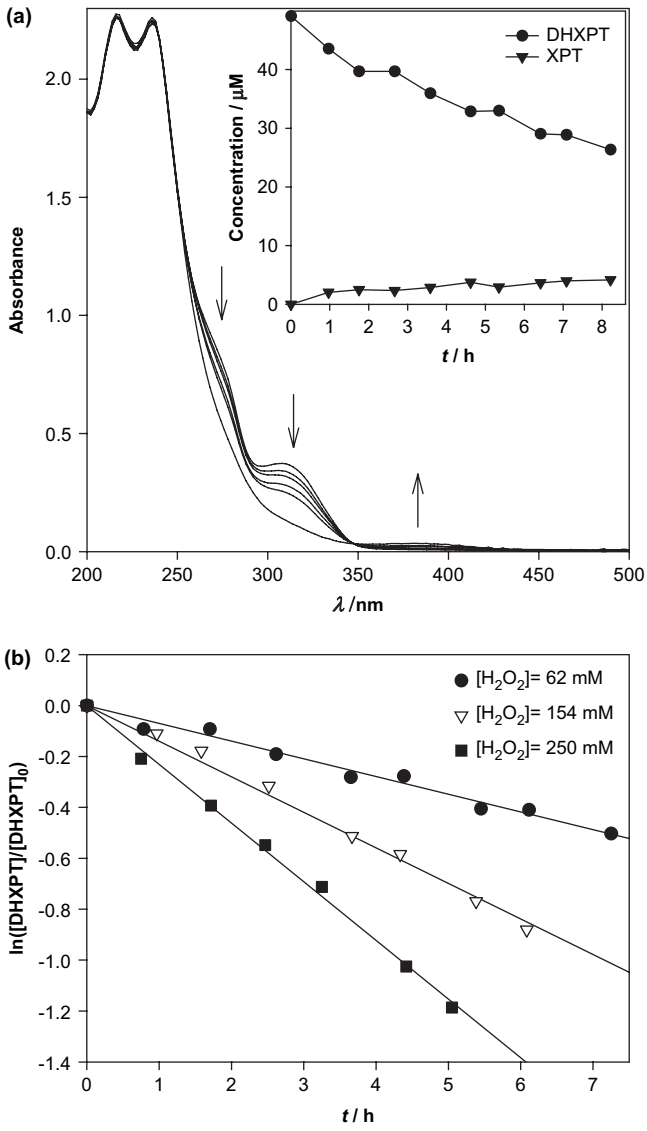


Figure 6. Reaction between DHXPT and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of product (XPT). Initial concentrations: $[DHXPT]_0$=50 μM, $[H_2O_2]_0$=62 mM. (b) First-order plots of the oxidation of DHXPT by H_2O_2 at different concentrations (Eq. 4, Section 4). DHXPT and XPT concentrations determined by HPLC analyses.

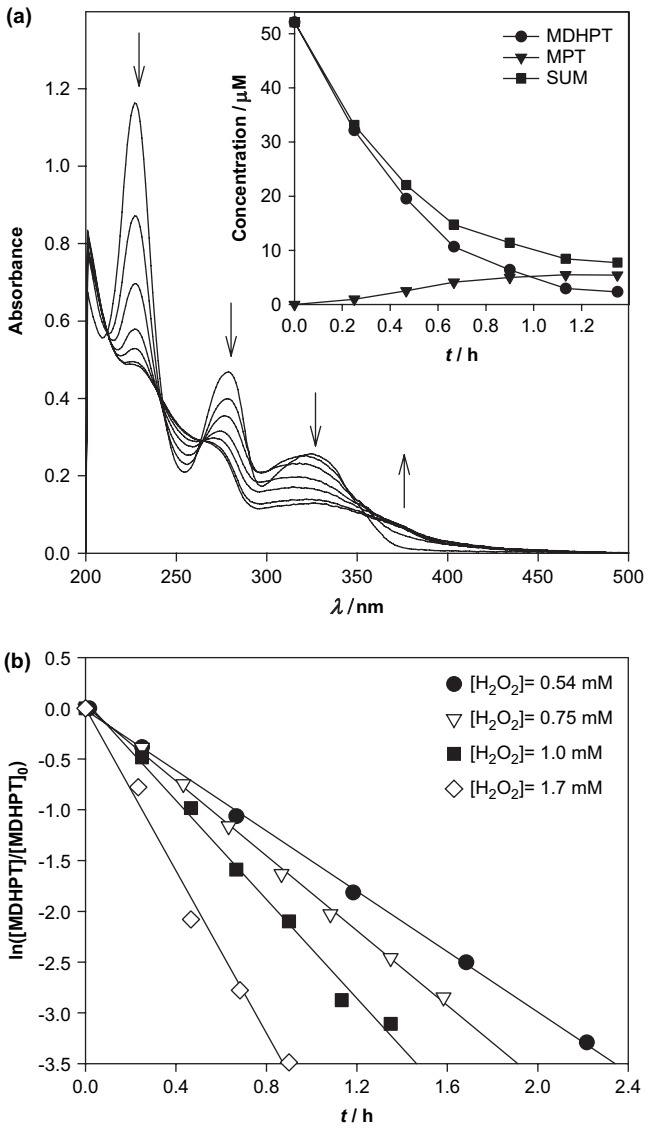


Figure 7. Reaction between MDHPT and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of product (MPT). Initial concentrations: $[MDHPT]_0$=54 μM, $[H_2O_2]_0$=1.0 mM. (b) First-order plots of the oxidation of MDHPT by H_2O_2 at different concentrations (Eq. 4, Section 4). Concentrations determined by HPLC analysis.

with MDHPT and DMDHPT, respectively (Figs. 7a and 8a; Scheme 3, pathway a). However, the concentration profiles show that only a fraction of the dihydropterin consumed is transformed into the corresponding oxidized derivative (Figs. 7a and 8a). For MDHPT, about 10% of the reactant is oxidized to MPT, whereas for DMDHPT only about 5% of the reactant is oxidized to DMPT.

Besides oxidized pterins, several additional products were detected by HPLC analysis, all of them having retention times lower than those corresponding to both reduced and oxidized pterins. They must therefore be very polar substances, most probably because of incorporation of oxygen into their structures. Spectral analysis of these additional products formed after long reaction times (t>5 h) suggested that the pterin moiety was oxidized and cleaved, yielding a group of non-pterinic products. These non-pterinic substances cannot originate from the further oxidation of 6-methylpterin and 6,7-dimethylpterin because these compounds are stable.

Therefore, at least two reaction pathways have to be considered for the reaction of H_2O_2 with MDHPT and DMDHPT: oxidation of the dihydropyrazine ring yielding the aromatic pyrazine moiety (Scheme 3, pathway a) and oxidation and cleavage of the dihydropterin to yield non-pterinic substances (Scheme 3, pathway b). This pattern was already observed for oxidation by O_2,[9] although the rate of this latter reaction is much slower.

2.5. Rate constants of the chemical reaction between H_2O_2 and dihydropterin derivatives

The apparent pseudo-first-order rate constants of the chemical reaction between H_2O_2 and several dihydropterin derivatives (k_{app}) in H_2O at pH 7.0±0.1 were determined. Each solution containing a given dihydropterin derivative and H_2O_2 was incubated at 37 °C and monitored as function of time by means of HPLC and spectral analyses. Under the experimental conditions used, disappearance

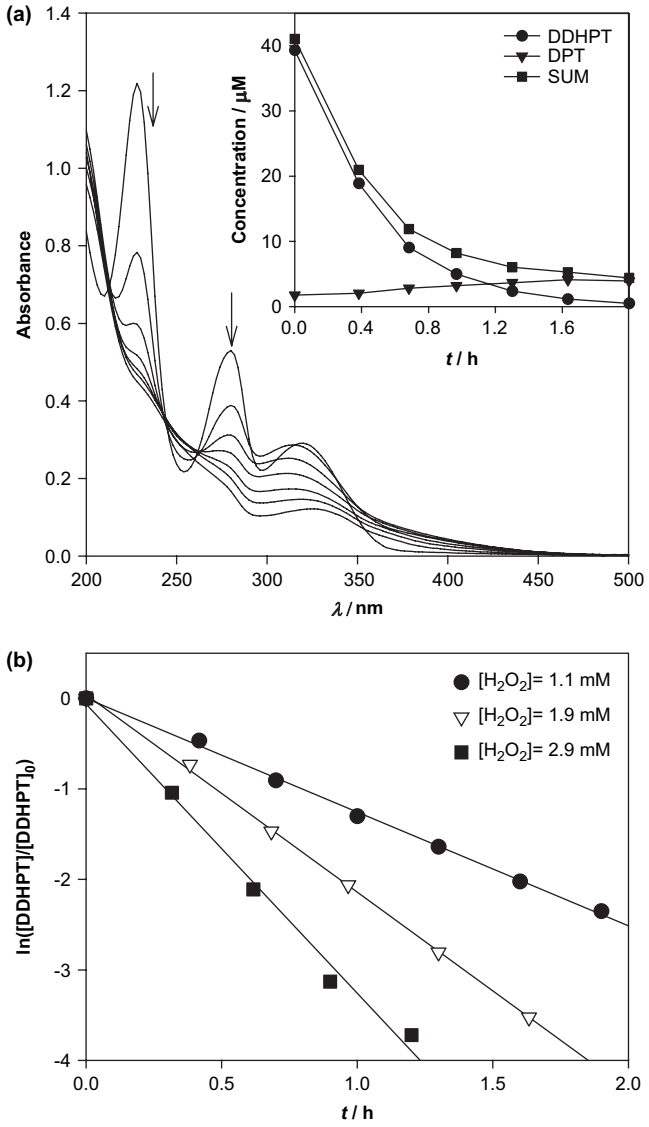
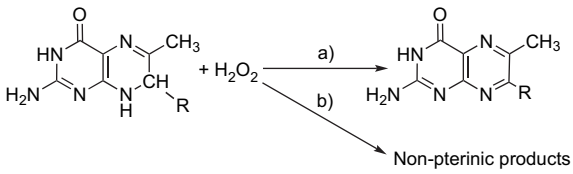


Figure 8. Reaction between DMDHPT and H_2O_2 (T=37 °C, pH=7.1±0.1). (a) Evolution of the absorption spectra as a function of time; arrows indicate the changes observed at different wavelengths. Optical path length=1 cm. Inset: evolution of the consumption of reactant and generation of product (DMPT). Initial concentrations: $[DMDHPT]_0$=40 μM, $[H_2O_2]_0$=1.9 mM. (b) First-order plots of the oxidation of DMDHPT by H_2O_2 at different concentrations (Eq. 4, Section 4). Concentrations determined by HPLC analysis.

of the dihydropterins followed in all cases first-order kinetics (Figs. 2b, 3b, 4b, 5b, 6b, 7b, and 8b).

For each compound, series of experiments were performed at different H_2O_2 concentrations. In all cases, linear relationships between k_{app} and the H_2O_2 concentration were found (Fig. 9), thus revealing a first kinetic order for H_2O_2. As a result, the bimolecular rate constants (k) were determined from the slopes of the corresponding plots k_{app} versus $[H_2O_2]$. The results obtained (Table 1)



Scheme 3. Reaction between MDHPT (R: H) and DMDHPT (R: –CH_3) and H_2O_2 in neutral aqueous solution at 37 °C.

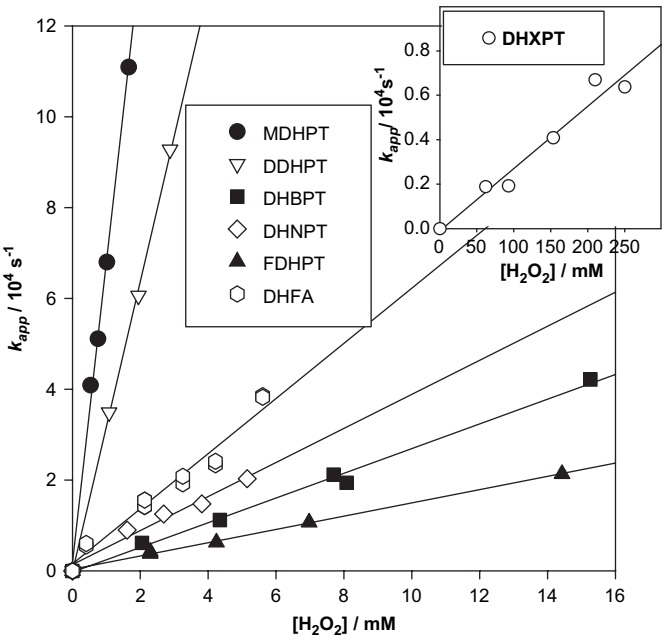


Figure 9. Chemical reaction between dihydropterins and H_2O_2 in neutral aqueous solution at 37 °C: plot of the apparent pseudo-first-order rate constants (k_{app}) as a function of H_2O_2 concentration.

revealed that the reactivity of dihydropterins toward H_2O_2 is strongly dependent on the chemical structure of their 6-substituent.

Comparison of k values indicates that the most reactive compounds within the studied series are MDHPT and DMDHPT. This result suggests that the higher electronic density on the pyrazine ring induced by the methyl group(s) favors the attack of H_2O_2. It is noteworthy that k is higher for MDHPT ((0.66±0.03) M^{-1} s^{-1}) than for DMDHPT ((0.32±0.02) M^{-1} s^{-1}), probably due to steric effects of the methyl group in C7. However, the same behavior was observed for the reaction between O_2 and dihydropterins.[9]

It is interesting to assess the reactivity of DHNPT, DHBPT, and DHFA, the most relevant derivatives from a biological point of view, toward H_2O_2. The k values reported in the present work show that the reaction of the mentioned derivatives with H_2O_2 will be significant if the H_2O_2 concentration is high. This is the case of vitiligo (vide supra), where millimolar concentrations of H_2O_2 has been reported.[12] Therefore, the oxidation of DHBPT, that accumulates in the skin of patients suffering vitiligo, should be taken into account.[11]

These results are in agreement with previous works reporting that these compounds, especially DHNPT, due to their reactivities toward reactive oxygen species, can be considered as antioxidants[18,19] and, consequently, might have a protective roll against oxidative stress. However, there are other studies that claim that when 6-formylpterin (FPT) is reduced by, for example, NADH the resulting 6-formyldihydropterin reacts with O_2 to yield H_2O_2.[20] In

Table 1
Rate constants of the chemical reaction between dihydropterins and H_2O_2 (k) in air-equilibrated aqueous solutions; pH=7.0±0.1; T=37 °C

Compound	k/M^{-1} s^{-1}
Dihydrobiopterin	$(2.7\pm0.2)\times10^{-2}$
Dihydroneopterin	$(3.7\pm0.4)\times10^{-2}$
6-Methyldihydropterin	0.66±0.03
6,7-Dimethyldihydropterin	0.32±0.02
Dihydroxanthopterin	$(2.5\pm0.2)\times10^{-4}$
Dihydrofolic acid	$(6.1\pm0.7)\times10^{-2}$
6-Formyldihydropterin	$(1.5\pm0.1)\times10^{-2}$

this case the dihydropterin derivative, that was not identified yet, would be acting as H_2O_2 generator and not as antioxidant. Such reduced pterin certainly is not 6-formyl-7,8-dihydropterin, which is rather stable in air-equilibrated solutions.[9] Alternatively, it could be a 5,8-dihydropterin derivative. It has been recently shown that these compounds, that can also be formed photochemically from oxidized pterins, react with O_2 very fast to yield H_2O_2.[21,22]

Although DHXPT also undergoes oxidation, its k value is much smaller than those determined for the other studied compounds. This fact is logical taking into account that in DHXPT the electronic density on the pyrazine ring is low due to the oxygen in position 6. Therefore, the electrophilic attack should be much less efficient than in the case of the rest of the series. It is also worth mentioning that since DHXPT is the product of the oxidation of those dihydropterins present in biological systems (DHBPT, DHNPT, and DHFA) and its oxidation is relatively slow, this compound might be produced and accumulated in vivo under oxidative conditions.

3. Conclusions

The chemical reaction between H_2O_2 and a group of 7,8-dihydropterins, one of the biologically active forms of pterins, has been investigated in aqueous solution at 37 °C, and physiological pH (~7). The series includes: dihydrobiopterin (DHBPT), dihydroneopterin (DHNPT), 6-formyldihydropterin (FDHPT), dihydrofolic acid (DHFA), dihydroxanthopterin (DHXPT), 6-methyldihydropterin (MDHPT), and 6,7-dimethyldihydropterin (DMDHPT) (Fig. 1). The rates of the reaction with H_2O_2, as well as the products formed, strongly depend on the chemical structure of the substituents.

The cleavage of the substituent and oxidation of C6 to yield DHXPT (>90%) is the main pathway of the reaction of DHNPT, DHBPT, and FDHPT with H_2O_2 (Scheme 1). These results show that biopterin (BPT), an oxidized pterin detected in the skin of patients suffering vitiligo, is not the product of the reaction between DHBPT and H_2O_2, as suggested in previous works.[14] The corresponding bimolecular rate constants (k) (Table 1) vary in the narrow range ($1.5–3.7\times10^{-2}$ M^{-1} s^{-1}) and reveal that the consumption of DHNPT, DHBPT, and FDHPT is significant in the presence of H_2O_2, at concentration of the same order of magnitude as that measured in tissues affected by vitiligo.[12]

The reaction between DHFA and H_2O_2 also leads to the formation of DHXPT as the main product (~60%) (Scheme 2, pathway a). However, FA (~40%) and a very small amount of FDHPT (<1%) were also detected (Scheme 2, pathways b and c, respectively). Oxidation of DHFA is important from a biological point of view since folic acid and its reduced forms are the most abundant pterin derivatives in living systems. The corresponding k value is of the same order of magnitude than those of the compounds described in the previous paragraph.

DHXPT also reacts with H_2O_2, the reaction is much slower, the corresponding k value being about 2 orders of magnitude smaller than those reported for the derivatives analyzed in the previous paragraphs (Table 1). Since DHXPT is the product of the oxidation of those dihydropterins present in biological systems (DHBPT, DHNPT, and DHFA) and its reaction with H_2O_2 is relatively slow, this compound might be produced and accumulated in vivo.

MDHPT and DMDHPT present the higher k values of the series (Table 1), which suggest that the higher electronic density on the pyrazine ring induced by the methyl group(s) favors the attack of H_2O_2. At least two reaction pathways have to be considered for the oxidation of these compounds: (a) oxidation of the dihydropyrazine ring yielding the aromatic pyrazine moiety, and (b) oxidation and cleavage of the dihydropterin molecule to yield non-pterinic substances (Scheme 3).

4. Experimental

4.1. General

Dihydropterins and the corresponding oxidized pterins were purchased from Schircks Laboratories, and used without further purification. Deionized water was further purified in a Milli Q Reagent Water System apparatus. The specific resistance measured was ~18 MΩ cm. H_2O_2 (30%) was from Merck. The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 to 2 M. The pH measurements were performed using a pH meter PHM220 (Radiometer Copenhagen) with a combined pH electrode pHC2011-8 (Radiometer Analytical).

4.2. UV–Vis analysis

Electronic spectra were recorded on a Cary-3 (Varian) spectrophotometer or on a diode array spectrophotometer 8452A (Hewlett Packard). Measurements were made in quartz cells of 1 cm optical path length. The absorption spectra of the solutions were recorded at regular intervals of time after mixing the reactants. 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 relative to the maximum absorbance 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 aqueous solutions of commercial standards. The comparison between NED and NRD spectra allows the characterization of the major products. The analysis based on these difference spectra is described elsewhere.[23,24]

4.3. High-performance liquid chromatography (HPLC)

A high-performance liquid chromatograph Prominence from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5 and UV/VIS photodiode array detector SPD-M20A) was employed for monitoring the reactions. A Pinnacle-II C18 column (250×4.6 mm, 5 μm; Restek) was used for product separation. Solutions containing 3–5% of acetronitrile and 95–97 of potassium phosphate aqueous solution (20 mM, pH 5.5) were used as eluent.

It should be noted that in the case of DHBPT, DHFA, and DHXPT the peak of the reactant could not be well separated from that of the corresponding product (DHXPT, FA, and XPT, respectively). Therefore, calibration curves were established and integrations of the peaks at different wavelengths were performed. Assuming that the peak considered is only due to the reactant and one known product, the concentration of both compounds can be calculated by resolving sets of equations as follows:

$$\text{Area}_{\lambda1} = f^{R}_{\lambda1}C^{R} + f^{P}_{\lambda1}C^{P} \quad (1)$$

$$\text{Area}_{\lambda2} = f^{R}_{\lambda2}C^{R} + f^{P}_{\lambda2}C^{P} \quad (2)$$

where $\text{area}_{\lambda1}$ and $\text{area}_{\lambda2}$ are the values resulting from integration of the chromatograms at analysis wavelengths $\lambda1$ and $\lambda2$, C^R and C^P are the concentrations of the reactant and the product, $f^R_{\lambda1}$, $f^P_{\lambda1}$, $f^R_{\lambda2}$, and

$f_{\lambda 2}^{P}$ are the factors obtained from the calibration curves for the reactant and the product at $\lambda 1$ and $\lambda 2$. Although only two equations are required for calculating C^{R} and C^{P}, more equations were used in order to check the results obtained.

4.4. Determination of the rate constant of the chemical reaction between H_2O_2 and dihydropterins

In all experiments the concentration of H_2O_2 was much higher than that of the studied dihydropterin ($[H_2O_2]>10[DHPT]$). Therefore, pseudo-first-order conditions for the consumption of dihydropterins can be assumed and the rate of disappearance of reactant should be given by the following pseudo-first-order rate equation:

$$-(d[DHPT]/dt) = k_{app}[DHPT] \quad (3)$$

Where [DHPT] is the concentration of a given dihydropterin derivative and k_{app} is the pseudo-first-order rate constant of the chemical reaction between H_2O_2 and DHPT. Therefore, integration of Eq. 3 leads to:

$$\ln([DHPT]/[DHPT]_0) = k_{app}t \quad (4)$$

and first-order kinetics should be observed for the disappearance of DHPT.

For determining k_{app}, air-equilibrated aqueous solutions (pH=7.1±0.1) of the dihydropterin derivatives were incubated at 37 °C, using a low temperature bath/circulator R1 (Grant Instruments) or a thermostat bath D8/17V (MGW Lauda). Consumption of the dihydropterin as a function of time was analyzed by HPLC (vide supra). k_{app} was calculated from the slope of the plot $\ln([DHPT]/[DHPT]_0)$ versus t.

If the reaction is of first order for H_2O_2, the rate of disappearance of a given dihydropterin derivative is given by Eq. 5,

$$-(d[DHPT]/dt) = k[DHPT][H_2O_2] \quad (5)$$

where k is the bimolecular rate constant of the chemical reaction between H_2O_2 and DHPT. Therefore, k can be determined from the slope of the plot k_{app} versus $[H_2O_2]$ (Eq. 6).

$$k_{app} = k[H_2O_2] \quad (6)$$

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