Stability of 7,8-Dihydropterins in Air-Equilibrated Aqueous Solutions

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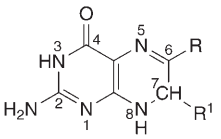
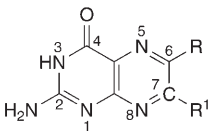
6-Substituted 7,8-dihydropterins (=2-amino-7,8-dihydropteridin-4(1*H*)-ones) are heterocyclic compounds that occur in a wide range of living systems and participate in relevant biological functions. In air-equilibrated aqueous solutions, these compounds react with dissolved O_2 (autooxidation). The rates of these reactions as well as the products formed strongly depend on the chemical structure of the substituents. 7,8-Dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin that bear electron-donor groups as substituents are the most reactive derivatives and undergo oxidation of the pterin moiety to yield the corresponding oxidized derivatives (6-methylpterin and 6,7-dimethylpterin, resp.). The oxidations of 7,8-dihydrobiopterin, 7,8-dihydroneopterin, and 7,8-dihydrofolic acid are slower, and they yield 7,8-dihydroxanthopterin as the main product. 7,8-Dihydroxanthopterin, 6-formyl-7,8-dihydropterin, and sepiapterin are rather stable, and their consumption in air-equilibrated solutions is negligible for several days. The pseudo-first-order rate constants of the reactions between these compounds and O_2 at 25° and 40° are reported. The biological implications of the results obtained are also discussed.

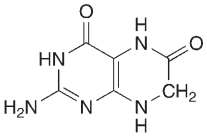
Introduction. – Pterins are a family of heterocyclic compounds that occur in a wide range of living systems and participate in relevant biological functions. The most common pterin derivatives are 6-substituted compounds. The molecular weight and functional groups of these substituents are quite different, *e.g.*, pterins may have substituents with one C-atom, with a short hydrocarbon chain, or larger substituents containing a 4-aminobenzoic acid moiety (*Fig. 1*). Derivatives with the latter type of substituents are frequently called conjugated pterins. Pterins can exist in different oxidation states and be divided into two classes according to this property: *a*) oxidized or aromatic pterins containing the pyrazine[2,3-*d*]pyrimidine ring structure and *b*) reduced pterins (*Fig. 1*). Within the latter group, 7,8-dihydropterins (=2-amino-7,8-dihydropteridin-4(1*H*)-ones) and 5,6,7,8-tetrahydropterins are the most important derivatives due to their biological activity, *e.g.*, 7,8-dihydroneopterin is secreted during the oxidative burst of stimulated macrophages [1], and 7,8-dihydrobiopterin and 5,6,7,8-tetrahydrobiopterin participate in the metabolism of amino acids [2]. The chemistry of 7,8-dihydropterins has been studied in detail [3].

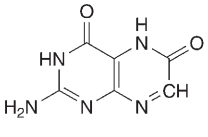
Depending on their oxidation state, pterins have totally different reactivities towards oxidizing agents, *e.g.*, whereas 7,8-dihydropterins are very efficient singlet-oxygen ($^1O_2$) *quenchers* [4], the reactivity of oxidized pterins towards $^1O_2$ is relatively low [5]. In air-equilibrated aqueous solutions, tetrahydropterins are rapidly oxidized by dissolved molecular oxygen in its ground-state (O_2), whereas 7,8-dihydropterins are





R	R^1	Dihydropterins	Oxidized pterins
$(CHOH)_2$–CH_2OH	H	7,8-Dihydroneopterin	Neopterin
$(CHOH)_2$–Me	H	7,8-Dihydrobiopterin	Biopterin
Me	H	7,8-Dihydro-6-methylpterin	6-Methylpterin
Me	Me	7,8-Dihydro-6,7-dimethylpterin	6,7-Dimethylpterin
PABA-Glu[a])	H	7,8-Dihydrofolic acid	Folic acid
CO–CHOH–Me	H	Sepiapterin	–
CHO	H	6-Formyl-7,8-dihydropterin	6-Formylpterin





7,8-Dihydroxanthopterin Xanthopterin

[a]) PABA-Glu =

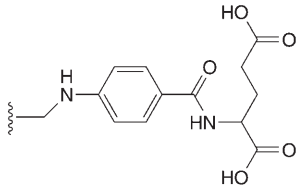


Fig. 1. *Molecular structures of the 7,8-dihydropterins investigated at physiological pH* (neutral forms), *together with the structures of corresponding oxidized pterins.* 7,8-Dihydro-6,7-dimethylpterin and 6,7-dimethylpterin have an additional Me group at C(7) of the pterin moiety.

relatively stable [6][7]. The oxidation of these compounds by dissolved O_2 or reactive oxygen species are of biological interest because they are generated under several pathological situations and accumulated in oxidative environments, *e.g.*, in the skin of patients affected by vitiligo [8][9]. However, despite the relevance of this process from the biological point of view, there is a lack of information on the chemical reaction between 7,8-dihydropterins and O_2 in aqueous solution at room or physiological temperature. Moreover, in several studies reported in the literature, experiments with these compounds are performed in the presence of O_2, and their oxidation is not controlled.

In this article, we describe the oxidation of a group of 6-substituted 7,8-dihydropterins, with substituents of different molecular weight and with different functional groups, by O_2 in air-equilibrated aqueous solutions. We investigated the following derivatives: 7,8-dihydro-6-methylpterin, 7,8-dihydro-6,7-dimethylpterin, 7,8-dihydrobiopterin, 7,8-dihydroneopterin, 6-formyl-7,8-dihydropterin, sepiapterin, 7,8-dihydroxanthopterin, and 7,8-dihydrofolic acid (*Fig. 1*). The pseudo-first-order rate

constants of the reaction between these compounds and O_2 at 25° and 40° were determined, and the products of the reactions were analyzed. We discuss the effects of the structural features of the dihydropterin derivatives and the biological implications of the results obtained.

Results and Discussion. – Aqueous solutions (pH 7.0–7.3) of 7,8-dihydropterins were prepared and kept in anaerobic conditions (Ar atmosphere) at 25° and 40° in order to check their stability in the absence of O_2. For all compounds studied, no significant changes in the absorption spectra of solutions after more than 48 h were observed. In the same way, HPLC measurements showed that the reactant concentrations did not decrease after 24 h of incubation in anaerobic conditions.

The chemical reactions between O_2 and 7,8-dihydropterins in air-equilibrated aqueous solution (pH 7.0–7.3) were investigated by UV/VIS spectrophotometry and HPLC analysis at 25° and 40°. The chemical changes were followed as a function of time during, at least, 24 h. The 'zero time' ($t = 0$) corresponds to the admission of air to the solution. In all cases, identification of products was carried out by means of HPLC analysis: retention times and spectra, obtained using the DAD detector, of samples and standard solutions were compared under different elution conditions.

Oxidation of 7,8-Dihydrobiopterin and 7,8-Dihydroneopterin. Significant spectral changes were observed in aqueous solutions of 7,8-dihydroneopterin at 40° after admission of air (*Fig. 2*). A blue-shift of *ca.* 20 nm was observed for the low-energy band. In addition, a very slight increase in the absorbance of the solution in the VIS region, with a maximum at *ca.* 423 nm, was also observed. On the other hand, slower spectral modifications were observed at 25° (data not shown). Nevertheless, the normalized experimental-difference (NED) spectra obtained at 25° and 40° were similar within the experimental error, suggesting that the product(s) of the reactions are the same at both temperatures. Very similar changes in absorption spectra, although much slower, were observed for 7,8-dihydrobiopterin (*Fig. 2*). Moreover, at 25° these changes were negligible (data not shown). This spectrophotometric analysis indicated that both compounds undergo similar chemical transformation, but at very different rates.

7,8-Dihydroxanthopterin was identified as the main product in the oxidation of 7,8-dihydroneopterin and 7,8-dihydrobiopterin, by means of HPLC analysis (*Fig. 3*, *a*). This observation is consistent with spectral analysis (*Fig. 2*): the low-energy absorption band of 7,8-dihydroxanthopterin is blue-shifted (λ_{max} *ca.* 310 nm) in comparison with the corresponding band of the reactants (λ_{max} *ca.* 330 nm).

The concentration profiles of reactants and 7,8-dihydroxanthopterin are shown in *Fig. 4*. For both compounds, the fraction of consumed reactant converted into 7,8-dihydroxanthopterin is more than 90%. Therefore, cleavage of the substituent and oxidation at C(6) is the main pathway of the reaction of 7,8-dihydroneopterin and 7,8-dihydrobiopterin with O_2 (*Scheme 1*, pathway *a*). These results are in agreement with previous studies that reported 7,8-dihydroxanthopterin as a typical oxidation product of 7,8-dihydropterin derivatives bearing a –CHOH– group at C(6) [3]. Taking into account the general mechanism proposed for the oxidation of these compounds [3][10], O_2 most likely reacts with the covalent hydrate form of the reactants. However, 6-formyl-7,8-dihydropterin was also detected by HPLC in the air-equilibrated solutions

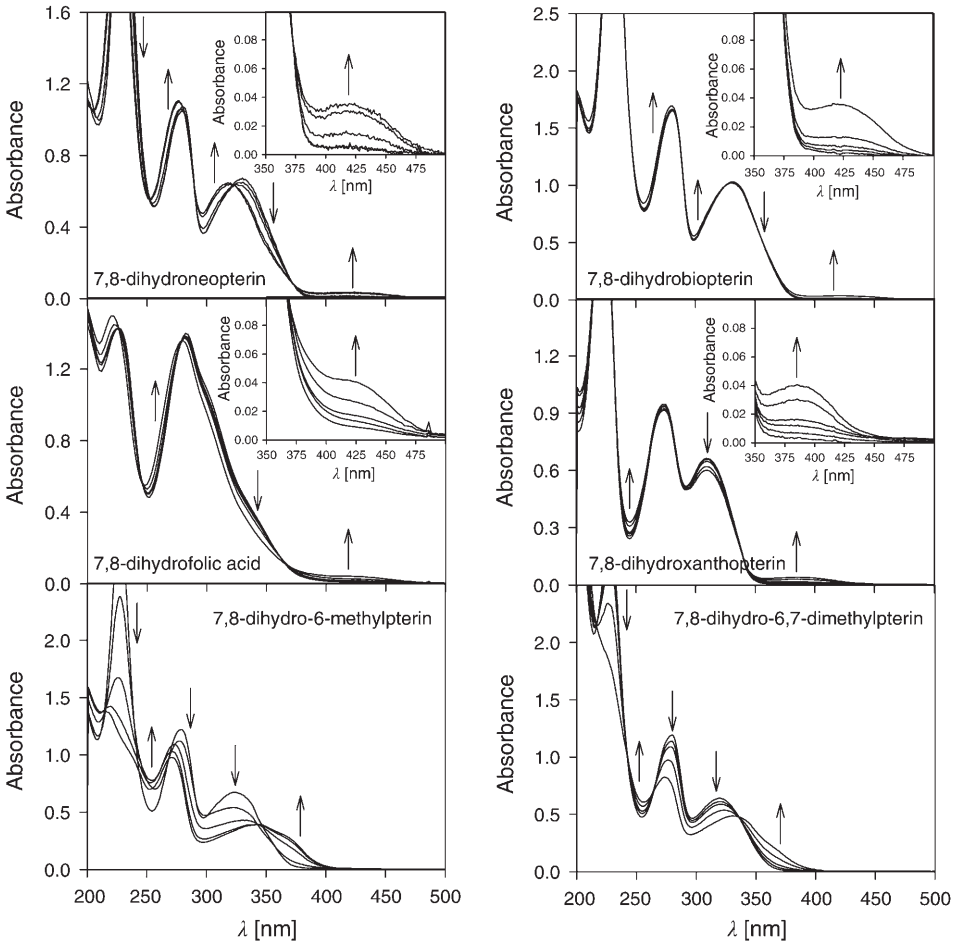


Fig. 2. *Oxidation of 7,8-dihydropterin derivatives in air-equilibrated aqueous solutions.* Evolution of the absorption spectra as a function of time. Spectra were recorded at 0, 2, 4.5, 9, and 24 h after admission of air for 7,8-dihydrobiopterin, 7,8-dihydrofolic acid, 7,8-dihydro-6-methylpterin, 7,8-dihydro-6,7-dimethylpterin; at 0, 2.5, 6, 21, and 25 h for 7,8-dihydroneopterin; and at 0, 1.33, 4, 7, 22, and 26 h for 7,8-dihydroxanthopterin. Arrows indicate the changes observed at different wavelengths. Optical path length 1 cm. $T = 40°$. Initial concentrations: $[7,8\text{-dihydroneopterin}]_0 = 107$ μM, $[7,8\text{-dihydrobiopterin}]_0 = 177$ μM, $[7,8\text{-dihydrofolic acid}]_0 = 66$ μM, $[7,8\text{-dihydro-6-methylpterin}]_0 = 120$ μM, $[7,8\text{-dihydro-6,7-dimethylpterin}]_0 = 104$ μM, $[7,8\text{-dihydroxanthopterin}]_0 = 96$ μM.

of 7,8-dihydroneopterin and 7,8-dihydrobiopterin (*Scheme 1*, pathway *b*; *Fig. 3*, *a*). The concentration of this product, although very low, increased as a function of time (*Fig. 4*). This result is also consistent with spectral analysis (*Fig. 2*), since the spectrum of 6-formyl-7,8-dihydropterin presents an absorption band in the VIS region (λ_{max} *ca.* 423 nm) [4]. Finally, no H_2O_2 was detected as a product of the studied reactions.

It is worth mentioning the biological implications of these results because both compounds can be produced in an oxidant environment [8][9]. Therefore, the

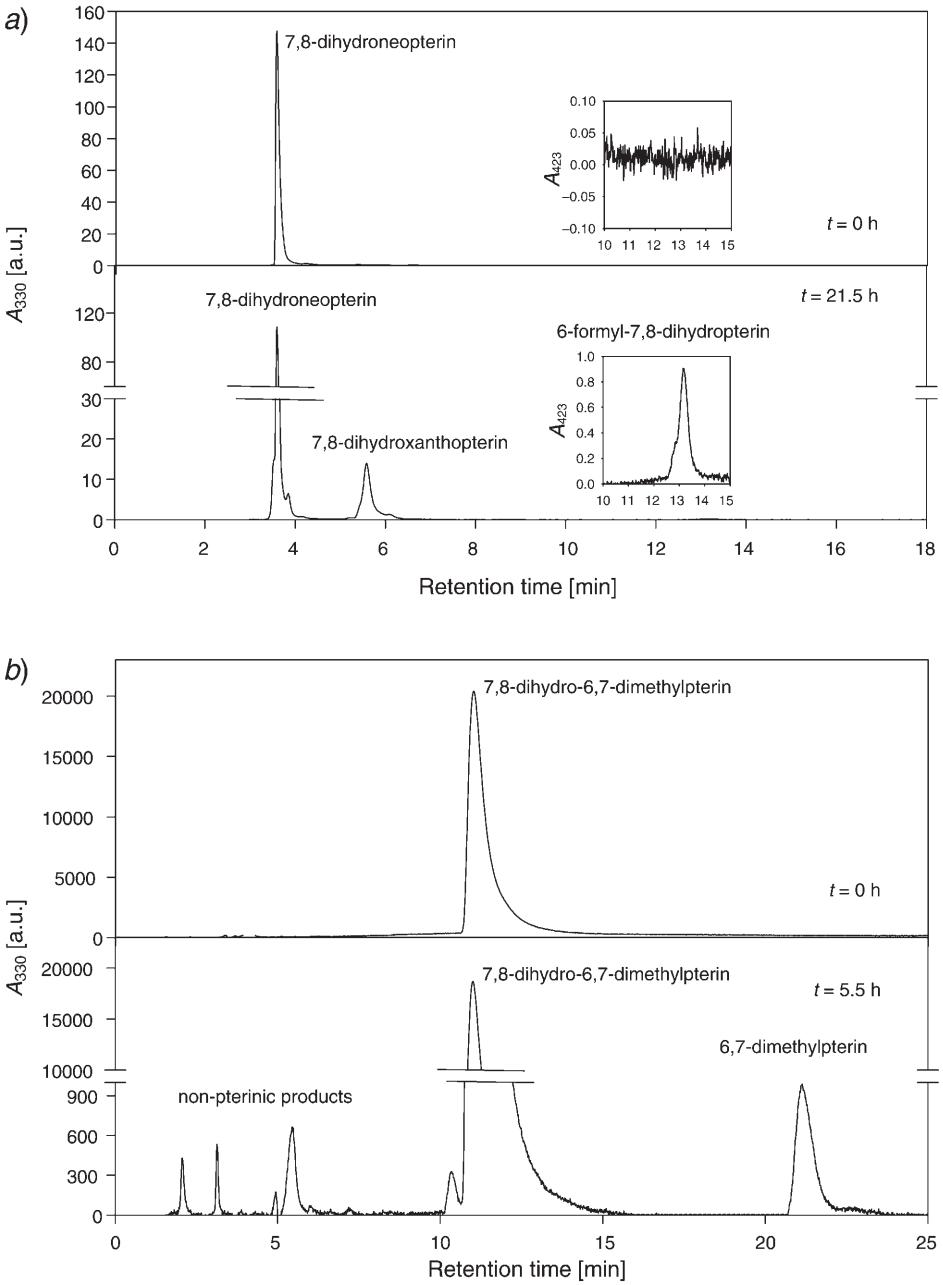
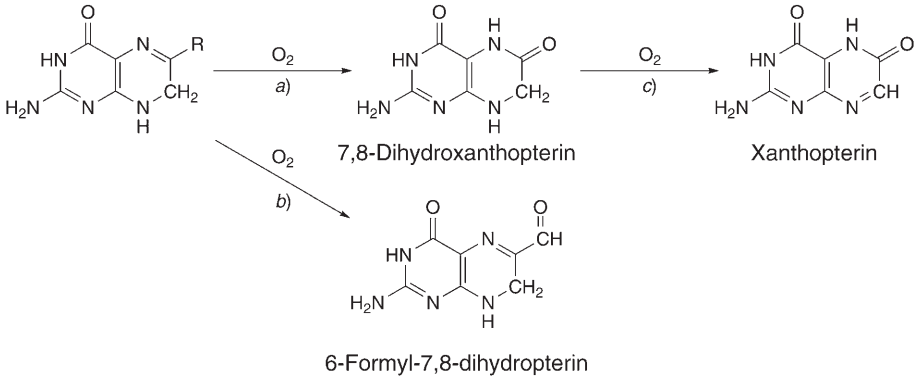


Fig. 3. *Chromatograms obtained in HPLC analysis:* a) *7,8-dihydroneopterin* (0 and 21.5 h after admission of air), b) *7,8-dihydro-6,7-dimethylpterin* (0 and 5.5 h after admission of air)

Scheme 1. *Oxidation of 7,8-Dihydroneopterin, 7,8-Dihydrobiopterin, and 7,8-Dihydrofolic Acid by O_2 in Neutral Aqueous Solution*



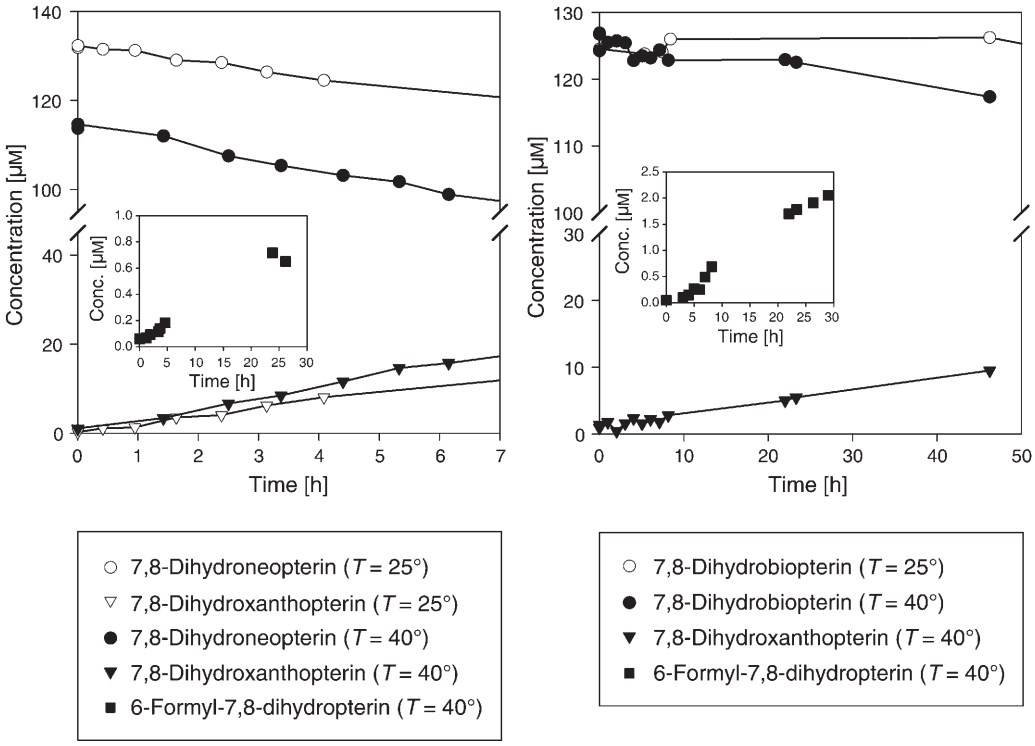


Fig. 4. *Oxidation of 7,8-dihydroneopterin and 7,8-dihydrobiopterin in air-equilibrated aqueous solution* (pH 7.2) *at 25° and 40°*. Evolution of the consumption of reactants and generation of products (7,8-dihydroxanthopterin and 6-formyl-7,8-dihydropterin). Concentrations determined by HPLC analysis.

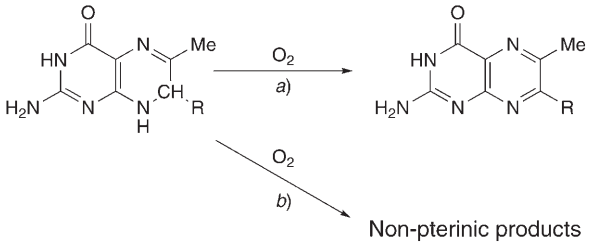
generation of 7,8-dihydroxanthopterin *in vivo via* the reactions described in this *Section* should be taken into account. It is also interesting to compare the process studied with

the oxidation by $^1O_2$. The reaction of 7,8-dihydrobiopterin and 7,8-dihydroneopterin with $^1O_2$ is very fast and leads to the oxidation of the pterin moiety, yielding the analogous oxidized pterin (biopterin and neopterin, resp.; *Fig. 1*), non-pterinic products, where the pterin moiety was oxidized and cleaved, and H_2O_2 [4].

Oxidation of 7,8-Dihydro-6-methylpterin and 7,8-Dihydro-6,7-dimethylpterin. Much faster spectral changes were observed when solutions of 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin were equilibrated with air at 25° and 40° (*Fig. 2*). In this case, the NED spectra obtained at 25° and 40° were also similar, suggesting that the reactions are the same at both temperatures (data not shown). The evolution of the spectra showed an absorbance increase at $\lambda > 340$ nm, where oxidized pterins show a typical band [11]. On the other hand, no absorbance increase was observed at λ *ca.* 310 nm (*Fig. 2*). This behavior indicates that the products of the reaction of 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin by O_2 are oxidized pterins, instead of 7,8-dihydroxanthopterin.

This hypothesis was confirmed by HPLC analysis: oxidation of 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin leads to 6-methylpterin and 6,7-dimethylpterin, respectively (*Fig. 3*,*b*; *Scheme 2*, pathway *a*). The reaction is faster for 7,8-dihydro-6-methylpterin (*Fig. 5*; *vide infra*). The Me group(s) seems to protect the oxidation at C(6) and, at the same time, makes the pterin moiety easier to oxidize. The concentration profiles show that only a fraction of the dihydropterin consumed is transformed into the corresponding oxidized derivative (*Fig. 5*). For 7,8-dihydro-6-methylpterin, *ca.* 50% of the reactant is oxidized to 6-methylpterin at both temperatures, whereas, for 7,8-dihydro-6,7-dimethylpterin, only *ca.* 20% of the reactant is oxidized to 6,7-dimethylpterin. Therefore, this reaction is not the only pathway responsible for the oxidation of 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin.

Scheme 2. *Oxidation of 7,8-Dihydro-6-methylpterin* (R = H) *and 7,8-Dihydro-6,7-dimethylpterin* (R = Me) *by O_2 in Neutral Aqueous Solution*



Besides oxidized pterins, several additional products were detected by HPLC analysis (*Fig. 3*,*b*), 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. Spectra of these additional products were recorded by using the DAD detector of the HPLC equipment. Such analyses, carried out especially for long times ($t > 20$ h), where conversion of the reactants was high, indicated that the pterin moiety was oxidized and cleaved, yielding a group of non-pterinic products, *i.e.*, the characteristic absorption bands of pterins and

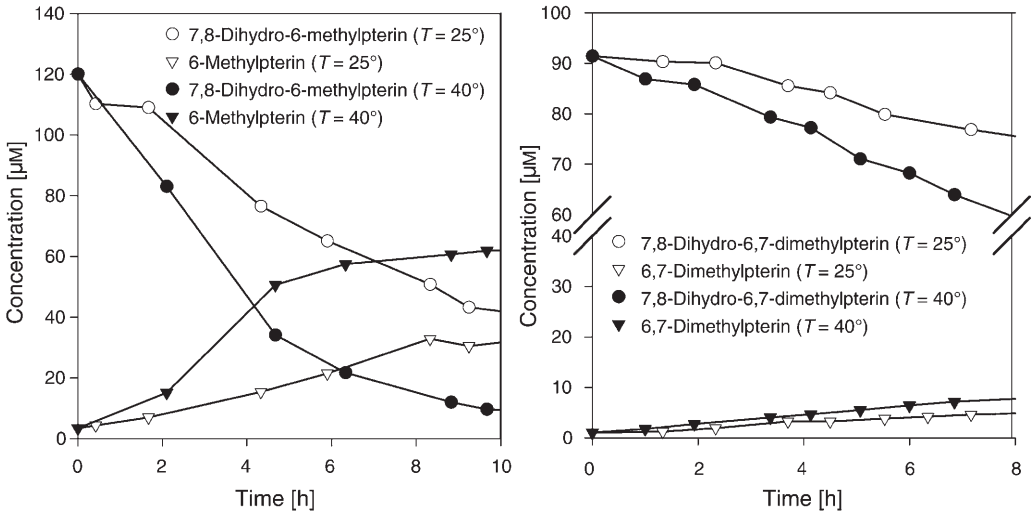


Fig. 5. *Oxidation of 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin in air-equilibrated aqueous solution* (pH 7.2) *at 25° and 40°*. Evolution of the consumption of reactants and production of the corresponding oxidized pterins (6-methylpterin and 6,7-dimethylpterin). Concentrations determined by HPLC analysis.

7,8-dihydropterins in the UV-A disappeared. 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 O_2 with 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin: oxidation of the dihydropyrazine ring yielding the aromatic pyrazine moiety (*Scheme 2*, pathway *a*), and oxidation and cleavage of the dihydropterin to yield non-pterinic substances (*Scheme 2*, pathway *b*). In this case, no H_2O_2 was detected.

Oxidation of 7,8-Dihydrofolic Acid. The spectra of solutions of 7,8-dihydrofolic acid change very slowly after admission of air (*Fig. 2*). Small changes in the absorption bands of 7,8-dihydrofolic acid and a very slight increase in the absorbance in the VIS region, with a maximum at *ca.* 423 nm, were observed. HPLC Analysis revealed that the main product of the oxidation of 7,8-dihydrofolic acid is 7,8-dihydroxanthopterin (*Scheme 1*, pathway *a*). Therefore, this compound seems to be the major oxidation product of the autooxidation of many 7,8-dihydropterin derivatives. The analysis of the corresponding concentration profiles (*Fig. 6*) showed that the fraction of consumed reactant converted into 7,8-dihydroxanthopterin is more than 80%. 6-Formyl-7,8-dihydropterin was also detected in the air-equilibrated solutions of 7,8-dihydrofolic acid (*Scheme 1*, pathway *b*). The concentration of this product, although very low, increased as a function of time (*Fig. 6*). This result is also consistent with spectral analysis (*Fig. 2*). Finally, no H_2O_2 was detected as a product of the studied reactions. Oxidation of 7,8-dihydrofolic acid is important from a biological point of view, since folic acid and its reduced forms are the most abundant pterin derivatives in living systems.

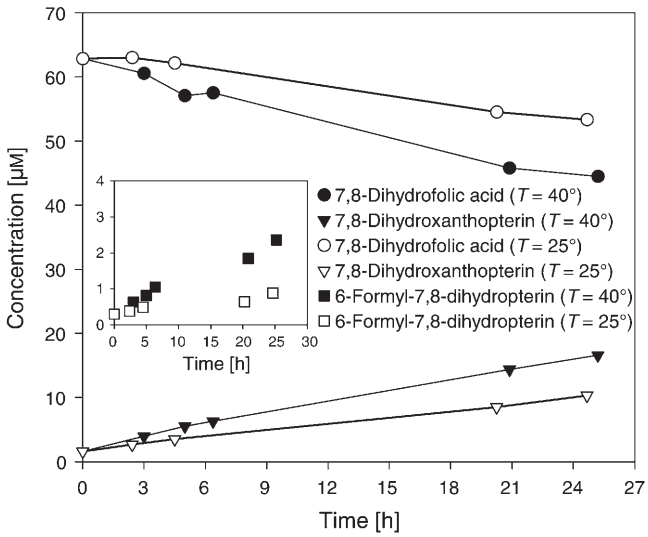


Fig. 6. *Oxidation of 7,8-dihydrofolic acid in air-equilibrated aqueous solution* (pH 7.2) *at 25° and 40°*. Evolution of the consumption of reactant and generation of products (7,8-dihydroxanthopterin and 6-formyl-7,8-dihydropterin). Concentrations determined by HPLC analysis.

7,8-Dihydroxanthopterin. Since 7,8-dihydroxanthopterin is the main product of the oxidation of the most important dihydropterins in biological systems (7,8-dihydroneopterin, 7,8-dihydrobiopterin, and 7,8-dihydrofolic acid), the stability of this compound in air-equilibrated aqueous solutions was also investigated. The spectral changes observed in the presence of air were very small at 40° (*Fig. 2*) and negligible at 25° (data not shown). However, the appearance of a band with maximum at *ca.* 390 nm was registered (*Fig. 2*). This observation together with the analysis of NED spectra suggests the formation of xanthopterin, the analogously oxidized form of the reactant (*Fig. 1*).

This hypothesis was confirmed by HPLC analysis: oxidation of 7,8-dihydroxanthopterin leads to xanthopterin (*Scheme 1*, pathway *c*). This reaction is similar to those observed in the cases of 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin; however, as revealed by concentration profiles obtained by means of HPLC analysis (*Fig. 7*), the oxidation is much slower (*vide infra*). In addition, the conversion of 7,8-dihydroxanthopterin into the corresponding oxidized form is complete; *i.e.*, the amount of xanthopterin produced is equal, within the experimental error, to the consumption of the reactant. In agreement with this result, no additional products were detected by HPLC analysis.

6-Formyl-7,8-dihydropterin and Sepiapterin. No modification of the spectra and no consumption measured by HPLC were registered for 6-formyl-7,8-dihydropterin and sepiapterin after 48 h of incubation at 25° and 40° in the presence of O_2. The C=O group in the substituent seems to make the chemical structure less reactive.

Rate of the Chemical Reaction between O_2 and 7,8-Dihydropterin Derivatives. The apparent pseudo-first-order rate constants of the chemical reaction between O_2 and several 7,8-dihydropterin derivatives (k_{app}) in H_2O at pH 7.0 ± 0.1 were determined,

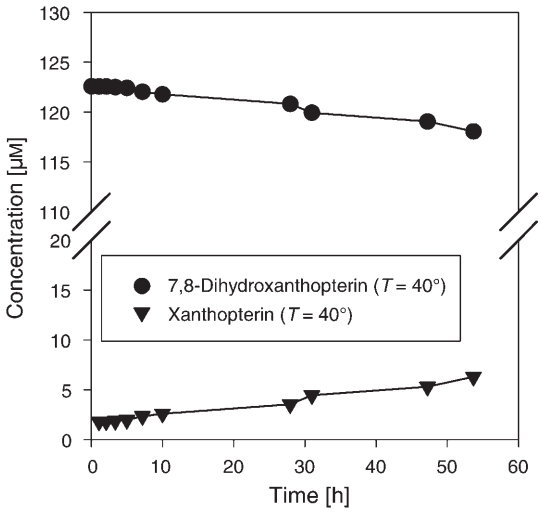


Fig. 7. *Oxidation of 7,8-dihydroxanthopterin in air-equilibrated aqueous solution* (pH 7.2) *at 40°*. Evolution of the consumption of reactant and generation of product (xanthopterin). Concentrations determined by HPLC analysis.

from HPLC analyses of the disappearance of the reactants during the incubation of the air-equilibrated aqueous solutions at 25° and 40°. Under the experimental conditions used, the disappearance of the 7,8-dihydropterins followed in all cases first-order kinetics (*Fig. 8*). The results obtained (*Table*) revealed that the reactivity of 7,8-dihydropterins towards O_2 is strongly dependent on the chemical structure of the substituent at C(6).

Taking into account that the chemical structure of 7,8-dihydrobiopterin and 7,8-dihydroneopterin differs only in a OH group (*Fig. 1*), and the oxidation process is exactly the same (*Scheme 1*), it seems to be surprising that the k_{app} values are quite different, 7,8-dihydroneopterin being much more easily oxidizable by O_2 than 7,8-dihydrobiopterin. Nevertheless, 7,8-dihydroneopterin has been reported to be especially reactive and very effective in scavenging radicals [12]. Moreover, it has been observed that 7,8-dihydroneopterin reacts with reactive species, such as $^1O_2$ [4] and the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) [13], faster than 7,8-dihydrobiopterin. Their different biological functions is maybe related to the very different reactivity of both compounds, *i.e.*, whereas 7,8-dihydrobiopterin acts as a cofactor in several metabolic reactions [2][14], 7,8-dihydroneopterin does not participate in enzymatic reactions and is synthesized mainly in activated macrophages during cell-mediated immune responses [1][15].

For 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin, the k_{app} values are relatively high, which suggests that the higher electronic density on the pyrazine ring induced by the Me group(s) favors the electrophilic attack of O_2. The same behavior was observed for the reaction between $^1O_2$ and oxidized pterins: 6,7-dimethylpterin and 6-methylpterin have the highest rate constant within a series of 6-substituted unconjugated pterin derivatives [5][16]. However, it is noteworthy that the

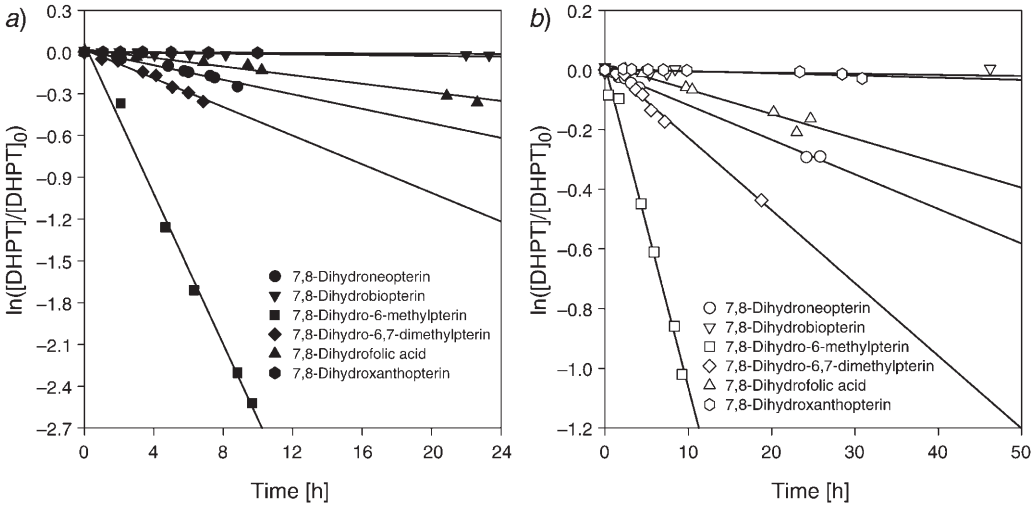


Fig. 8. *First-order plots of the oxidation of the 7,8-dihydropterin derivatives* (DHPT) *in air-equilibrated aqueous solutions* (*Eqn. 2*, *Exper. Part*). Concentrations of dihydropterins determined by HPLC analysis, pH 7.0 ± 0.1. *a*) $T = 40°$. *b*) $T = 25°$.

Table. *Apparent Pseudo-First-Order Rate Constants of the Chemical Reaction between 7,8-Dihydropterins and O_2 (k_{app}) and Half Life ($t_{1/2}$) of 7,8-Dihydropterins in Air-Equilibrated Aqueous Solutions.* pH 7.0 ± 0.1.

Compound	$T = 25°$		$T = 40°$	
	k_{app} [10^{-2} h^{-1}]	$t_{1/2}$ [h]	k_{app} [10^{-2} h^{-1}]	$t_{1/2}$ [h]
7,8-Dihydrobiopterin	0.04 ± 0.02	*ca.* 1700	0.13 ± 0.02	*ca.* 530
7,8-Dihydroneopterin	1.2 ± 0.3	*ca.* 60	2.3 ± 0.3	30
7,8-Dihydro-6-methylpterin	11 ± 1	6.5	27 ± 2	2.6
7,8-Dihydro-6,7-dimethylpterin	2.5 ± 0.2	28	5.4 ± 0.4	13
7,8-Dihydroxanthopterin	0.05 ± 0.02	*ca.* 1400	0.11 ± 0.04	*ca.* 630
7,8-Dihydrofolic acid	0.8 ± 0.1	*ca.* 90	1.5 ± 0.6	46
6-Formyl-7,8-dihydropterin	< 0.02	> 3000	< 0.02	> 3000
Sepiapterin	< 0.02	> 3000	< 0.02	> 3000

k_{app} value is higher for 7,8-dihydro-6-methylpterin than for 7,8-dihydro-6,7-dimethylpterin. Taking into account that the reaction occurs on the C(7)–C(8) bond, steric effects due to the Me group at C(7) of 7,8-dihydro-6,7-dimethylpterin might hinder the attack of O_2 onto the pyrazine ring.

Although 7,8-dihydroxanthopterin also undergoes the oxidation of the pyrazine ring to yield the corresponding oxidized derivative xanthopterin, its k_{app} value is much smaller than those determined for 7,8-dihydro-6-methylpterin and 7,8-dihydro-6,7-dimethylpterin. However, this observation is expected taking into account the hypothesis proposed in the previous paragraph; *i.e.*, in xanthopterin, the electronic density on the pyrazine ring is low due to the O-atom at C(6). Therefore, the electrophilic attack of O_2 should be much less efficient than in the case of 7,8-dihydro-

6-methylpterin and 7,8-dihydro-6,7-dimethylpterin. It is also worth mentioning that, since 7,8-dihydroxanthopterin is the product of the oxidation of those dihydropterins present in biological systems (7,8-dihydrobiopterin, 7,8-dihydroneopterin, and 7,8-dihydrofolic acid) and its oxidation is relatively slow, this compound and xanthopterin might be produced *in vivo* under oxidative conditions.

Finally, taking into account the k_{app} values, the half life ($t_{1/2}$), the time required for the concentration of a given 7,8-dihydropterin to decay to half of its initial value, was calculated at 25° and 40° for each studied compound (*Eqn. 3* in *Exper. Part* and *Table*). These $t_{1/2}$ values provide a direct evaluation of the stability of air-equilibrated aqueous solutions of 7,8-dihydropterins and should be taken into account when experiments with these compounds are carried out in air-atmosphere.

Conclusions. – The chemical reaction between dissolved O_2 and a group of 7,8-dihydropterins, one of the biologically active forms of pterins, have been investigated in aqueous solution at 25° and 40°, and physiological pH (*ca.* 7). The series includes 7,8-dihydro-6-methylpterin, 7,8-dihydro-6,7-dimethylpterin, 7,8-dihydrobiopterin, 7,8-dihydroneopterin, 6-formyl-7,8-dihydropterin, sepiapterin, 7,8-dihydroxanthopterin, and 7,8-dihydrofolic acid. Whereas 7,8-dihydropterins are stable under anaerobic conditions, the rates of the reaction with O_2 as well as the oxidation products strongly depend on the chemical structure of the substituents.

The oxidations of 7,8-dihydrobiopterin, 7,8-dihydroneopterin, and 7,8-dihydrofolic acid yield 7,8-dihydroxanthopterin as the main oxidation product (> 80%) and 6-formyl-7,8-dihydropterin as a minor product (*Scheme 1*). The values of the apparent pseudo-first-order rate constant (k_{app}) revealed significant differences in the chemical reactivity toward O_2. Considering that the chemical structure of 7,8-dihydrobiopterin and 7,8-dihydroneopterin differs only in a OH group (*Fig. 1*), and the oxidation process is exactly the same, it is noteworthy that k_{app}(7,8-dihydroneopterin) $\gg k_{app}$(7,8-dihydrobiopterin). The half-life values ($t_{1/2} = \ln 2/k_{app}$; *Table*) show that, in experiments performed with 7,8-dihydroneopterin and 7,8-dihydrofolic acid, two important pterin derivatives in biological systems, the autooxidation of these compound should be controlled.

7,8-Dihydro-6-methylpterin and 7,8-Dihydro-6,7-dimethylpterin present the higher k_{app} values of the series (*Table*), which suggests that the higher electronic density on the pyrazine ring induced by the Me group(s) favors the electrophilic attack of O_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 2*).

Finally, 7,8-dihydroxanthopterin, 6-formyl-7,8-dihydropterin, and sepiapterin are the most stable compounds of this series, and their consumption in air-equilibrated solutions is negligible for several days. Since 7,8-dihydroxanthopterin is the product of the oxidation of those dihydropterins present in biological systems (7,8-dihydrobiopterin, 7,8-dihydroneopterin, and 7,8-dihydrofolic acid), and its oxidation to xanthopterin (*Scheme 1*) is relatively slow, this compound and xanthopterin might be produced *in vivo* under oxidative conditions.

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Experimental Part

General. 7,8-Dihydropterins and the corresponding oxidized pterins were purchased from *Schircks Laboratories*, and used without further purification. The pH of the aq. solns. 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 2M. The pH measurements were performed with a pH-meter *PHM220* (*Radiometer Copenhagen*) and a combined pH electrode *pHC2011-8* (*Radiometer Analytical*).

To check the oxidation of 7,8-dihydropterins during preparation and manipulation of solns., control solns. were prepared under anaerobic conditions (Ar atmosphere), using a glovebox. The O_2 concentration in the H_2O employed for preparing the 7,8-dihydropterin solns. within the air-free compartment was monitored using an O_2-selective electrode (*Celiox 325*) and kept below 0.02 ppm. The same experimental setup was used to control the stability of the studied compounds in the absence of O_2.

Determination of the Apparent First-Order Rate Constant of the Chemical Oxidation of 7,8-Dihydropterins by O_2. In all experiments, the concentration of O_2 was higher than that of the studied dihydropterin. In addition, the reactions took place in open cells with stirring. Taking into account these experimental conditions, and that the consumption of O_2 was in all cases relatively slow, the O_2 concentration can be considered constant during the experiments. Therefore, pseudo-first-order conditions for the consumption of dihydropterins can be assumed, and the rate of consumption of reactant should be given by the following pseudo-first-order rate equation:

$$-(d[\mathrm{DHPT}]/dt) = k_{\mathrm{app}}\,[\mathrm{DHPT}] \tag{1}$$

where [DHPT] is the concentration of a given 7,8-dihydropterin derivative, and k_{app} is the pseudo-first-order rate constant of the chemical reaction between O_2 and DHPT. Therefore, integration of *Eqn. 1* leads to:

$$\ln([\mathrm{DHPT}]/[\mathrm{DHPT}]_0) = k_{\mathrm{app}}\,t \tag{2}$$

and first-order kinetics should be observed for the disappearance of DHPT. For determining k_{app}, air-equilibrated aq. solns. (pH 7.1 ± 0.1) of the 7,8-dihydropterin derivatives were incubated at 25° and 40°, using a low-temp. 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 infra*). k_{app} was calculated from the slope of the plot $\ln([\mathrm{DHPT}]/[\mathrm{DHPT}]_0)$ *vs. t*.

The half life ($t_{1/2}$), the time taken for the concentration of a reactant to fall to half of its initial value, was calculated using *Eqn. 3*.

$$t_{1/2} = \ln 2/k_{\mathrm{app}} \tag{3}$$

High-Performance Liquid Chromatography. High-performance liquid chromatography (HPLC; *System Gold* chromatographic system from *Beckman Instruments*) was employed for monitoring the reaction. A *Pinnacle-II C18* column (250 × 4.6 mm, 5 μm; *Restek*) was used for product separation. Solns. containing 3–5% of MeCN and 95–97 of potassium phosphate aq. soln. (20 mM, pH 5.5) was used as eluent. HPLC Runs were monitored with a UV/VIS photodiode array detector (*SPD-M20A*, *Shimadzu*).

It should be noted that, in the case of 7,8-dihydrobiopterin and 7,8-dihydroxanthopterin, the peak of the reactant could not be well separated from that of the corresponding product (7,8-dihydroxanthopterin and xanthopterin, resp.). 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:

$$\text{Area}_{\lambda 1} = f^{R}_{\lambda 1}\, C^{R} + f^{P}_{\lambda 1}\, C^{P} \tag{4}$$

$$\text{Area}_{\lambda 2} = f^{R}_{\lambda 2}\, C^{R} + f^{P}_{\lambda 2}\, C^{P} \tag{5}$$

where $\text{Area}_{\lambda 1}$ and $\text{Area}_{\lambda 2}$ are the values resulting from integration of the chromatograms at analysis wavelengths $\lambda 1$ and $\lambda 2$, and C^R and C^P are the concentrations of the reactant and the product, respectively, $f^{R}_{\lambda 1}, f^{P}_{\lambda 1}, f^{R}_{\lambda 2}, f^{P}_{\lambda 2}$ 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.

UV/VIS Analysis. Electronic spectra were recorded on a *Varian Cary-3* spectrophotometer. Measurements were conducted in quartz cells of 1-cm optical-path length. The absorption spectra of the solns. were recorded at regular intervals of time after admission of air, and the signals were averaged and smoothed with the *Varian* software. Experimental-difference (ED) spectra were obtained by subtracting the spectrum at time $t = 0$ from the subsequent spectra recorded at different times t. Each ED spectrum was normalized relative to the maximum absorbance value of the absorbance difference, yielding the normalized experimental-difference (NED) spectrum.

Determination of the Concentration of H_2O_2. For the determination of H_2O_2, a *Cholesterol Kit* (*Wiener Laboratorios S. A. I. C.*) was used. H_2O_2 was quantified after reaction with 4-aminophenazone and phenol [17][18]. The main features of the method have already been described in [19].

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