Formation of Oxygen Radicals in Solutions of Different 7,8-Dihydropterins: Quantitative Structure-Activity Relationships

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Under certain conditions, 7,8-dihydroneopterin in aqueous solution promotes hydroxyl-radical formation. Thus, we investigated the stimulation of hydroxyl-radical formation by ten different 7,8-dihydropterins (=2-amino-7,8-dihydropteridin-4(1*H*)-one), *i.e.*, 6-(1′-hydroxy) derivatives **1** and **2**, methyl derivatives **3**–**7**, and 6-(1′-oxo) derivatives **8**–**10**. All but the 6-(1′-oxo) derivatives produced hydroxyl radicals, as measured by the amount of salicylic acid hydroxylation products. This amount was dependent on the stability of the dihydropterin used. In the presence of chelated iron ions, hydroxylation was increased in every case; even 6-(1′-oxo) derivatives showed a low hydroxylation of salicylic acid. The degree of increase, however, strongly depended on the side chain of the dihydropterin. The 7,8-dihydroneopterin (**2**) was investigated in more detail. Iron ions influenced both, the stability of **2** and hydroxyl-radical formation. While iron ions determined the kinetics of the reaction, the amount of **2** was responsible for the amount of hydroxyl radicals formed. Our data establish that promotion of hydroxyl-radical formation by 7,8-dihydropterins depends on the oxidizability of the dihydropterins and on their iron-chelating properties.

Introduction. – During the oxidative burst of stimulated macrophages, reactive O-species, as well as dihydroneopterin (=2-amino-7,8-dihydro-[(1*S*,2*R*)-1,2,3-trihydroxypropyl]pteridin-4(1*H*)-one; **2**) and neopterin, are secreted [1][2]. Neopterin is used as an indicator for the activation of the cellular immune system [3]. Nevertheless, the biological roles of neopterin and dihydroneopterin are unclear. Since about ten years ago, the interaction of pterins with radical-mediated reactions has been under investigation [4]. Reduced pterins were shown to act as scavengers of free radicals generated by different chemical or biological systems: *e.g.*, chemiluminescence of luminol induced by macrophages or chemical systems [5–8], toxicity of chloramine T or hydrogen peroxide against bacteria [9][10], low density lipoprotein oxidation [11], nitration of tyrosine by peroxynitrite [12], and electron-spin resonance (ESR)[1]) experiments with spin traps [13]. In all these experiments, pterins were exposed to preformed radicals, and the action of pterins on those radicals was investigated. In contrast, recently it was found that 7,8-dihydroneopterin (**2**) in high concentrations may enhance radical-mediated reactions like luminol chemiluminescence and apoptosis [14–16]. In addition, it was shown that, in solutions of **2**, hydroxyl radicals are formed in the absence of any radical source besides dissolved O_2 [17][18]. Hydroxyl-radical formation was dependent on the addition of chelated iron ions.

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[1]) Abbreviations: DHBA, 2,5-dihydroxybenzoic acid; EDTA (=H_4edta) ethylenediamine tetraacetic acid; ESR, electron spin resonance; PBS, phosphate-buffered saline.

The aim of this study was to compare the efficiency of different dihydropterins to produce hydroxyl radicals, and to establish a correlation with their structures. Furthermore, we intended to define in more detail the influence of iron ions on the interactions of dihydropterins and free radicals.

Results and Discussion. – In this study we investigated ten different 7,8-dihydropterins (see **1**–**10** in *Fig. 1*) for their stimulatory effect upon hydroxyl-radical formation, as measured by the hydroxylation of salicylic acid. The compounds tested can be divided into three groups: *i*) 6-(1′-hydroxy) derivatives **1** and **2**, *ii*) methyl

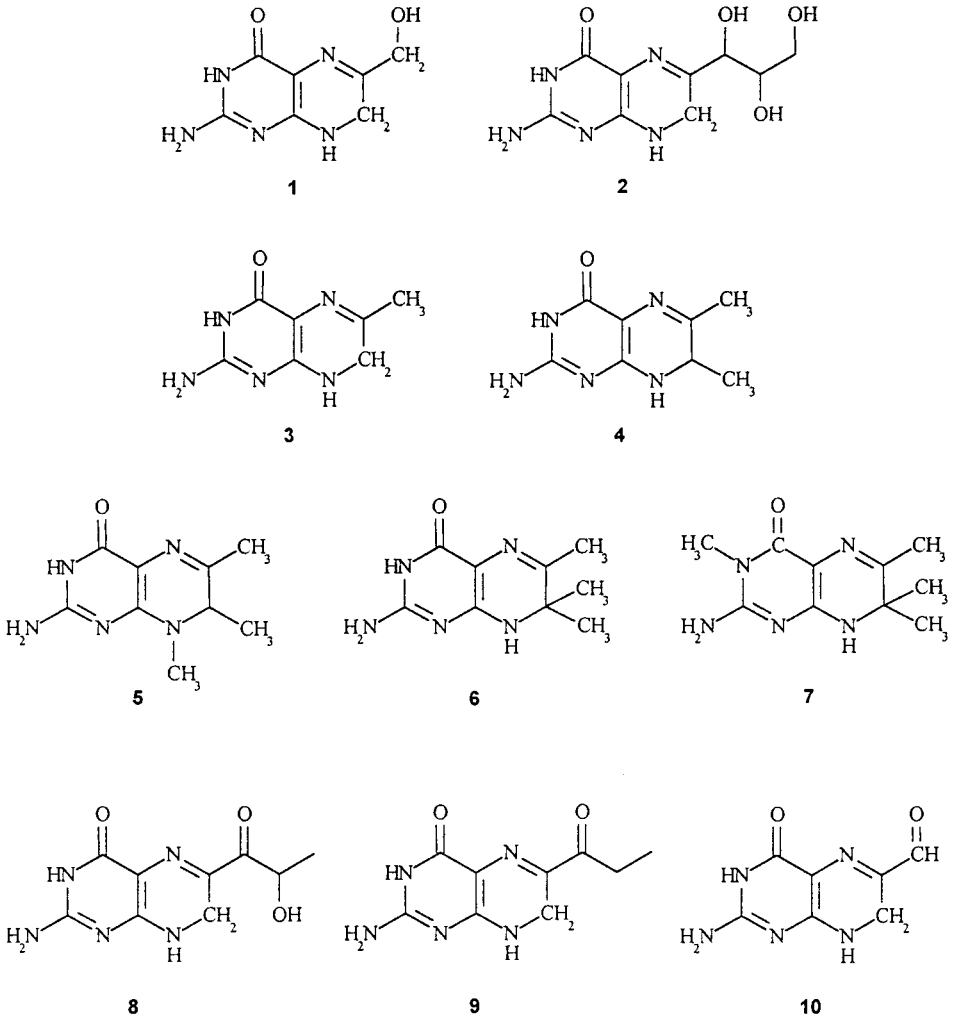

Fig. 1. *Structures of the studied dihydropterins: 7,8-dihydro-6-(hydroxymethyl)pterin* (**1**), *7,8-dihydroneopterin* (**2**), *7,8-dihydro-6-methylpterin* (**3**), *7,8-dihydro-6,7-dimethylpterin* (**4**), *7,8-dihydro-6,7,8-trimethylpterin* (**5**), *7,8-dihydro-6,7,7-trimethylpterin* (**6**), *7,8-dihydro-3,6,7,7-tetramethylpterin* (**7**), *sepiapterin* (**8**), *deoxysepiapterin* (**9**), *and 6-formyl-7,8-dihydropterin* (**10**)

derivatives **3**–**7** [19][20], and *iii*) 6-(1′-oxo) derivatives **8**–**10**. Time-dependent formation of salicylic acid hydroxylation products was followed up to *ca.* 18 h and occurred in all pterin solutions, except in those of the 1′-oxo derivatives **8**–**10** (*Fig. 2*, right-hand) which are rather stable compounds. The source of hydroxyl radicals is assumed to be superoxide, which could be generated from dissolved O_2 by the autooxidation of traces of metal ions or during dihydropterin oxidation and converted to hydroxyl radicals *via* the *Fenton* reaction catalyzed by trace iron. The formation of superoxide has been described to occur during oxidation of pterins, but only for fully reduced tetrahydropterins [21–23]. Moreover, we recently reported that, in solutions of 7,8-dihydroneopterin, hydroxyl-radical formation can be diminished by addition of desferrioxamine, suggesting that trace metal ions are the source of superoxide rather than dihydropterins [17].

Parallel to the measurement of hydroxylation products, the decay of the dihydropterins in solution was followed (*Fig. 2*, left-hand). The extent of hydroxylation after completion of the assay was correlated with the decay of the corresponding dihydropterin. A pattern of three different types of behavior according to the three classes of dihydropterins investigated can easily be seen in the diagram shown in *Fig. 3*,*a*. Methyl derivatives **3**–**7** show hydroxylation yields in accordance to the extent of the decay of the pterin, which varied considerably. The 6-(1′-oxo) derivatives **8**–**10** show essentially no hydroxylation, and 6-(1′-hydroxy) derivatives **1** and **2** give low yields of hydroxylation compared to their low stability. The decay of dihydropterins *vs.* time (*Fig. 2*) can be described by an exponential curve, and the decay constants of dihydropterins can be calculated (*Table*). These constants correlate with the amount of hydroxylation and show essentially the same behavior as demonstrated in *Fig. 3*,*a*.

Table. *Decay Constants of Different 7,8-Dihydropterins* [a])

	Decay constant [h^{-1}]	
	absence of Fe^{3+}/EDTA	presence of Fe^{3+}/EDTA
1 7,8-Dihydro-6-(hydroxmethyl)pterin	0.022 ± 0.007	0.035 ± 0.015
2 7,8-Dihydroneopterin	0.052 ± 0.011	0.037 ± 0.008
8 Sepiapterin	0.005 ± 0.002	0.004 ± 0.002
9 Deoxysepiapterin	0.007 ± 0.001	0.007
10 6-Formylpterin	0.016 ± 0.002	0.015 ± 0.006
3 7,8-Dihydro-6-methylpterin	0.108 ± 0.027	0.103 ± 0.031
4 7,8-Dihydro-6,7-dimethylpterin	0.029 ± 0.003	0.041 ± 0.005
5 7,8-Dihydro-6,7,8-trimethylpterin	0.041 ± 0.019	0.054 ± 0.025
6 7,8-Dihydro-6,7,7-trimethylpterin	0.016 ± 0.010	0.022 ± 0.011
7 7,8-Dihydro-3,6,7,7-tetramethylpterin	0.048 ± 0.017	0.054 ± 0.011

[a]) Dihydropterins (500 μM) were incubated in the presence of 500 μM salicylic acid and in the presence or absence of 20/40 μM ferric ions/EDTA. After 0, 5.8, 11.6, and 17.5 h, aliquots were analyzed by HPLC and the dihydropterin content measured. From the decrease of dihydropterin *vs.* time, the decay constants [h^{-1}] of the dihydropterins were calculated (mean values ± s.d. of 3 runs, except for **2** (5 runs) and **9** (1 run) in the presence of Fe^{3+}/EDTA).

Addition of chelated ferric ions to the assay mixtures influences both the stability of the compounds tested and hydroxylation (*Fig. 2*). While the effect on the stability ranges from a moderate decrease to a 1.5 fold increase of the decay with no specific

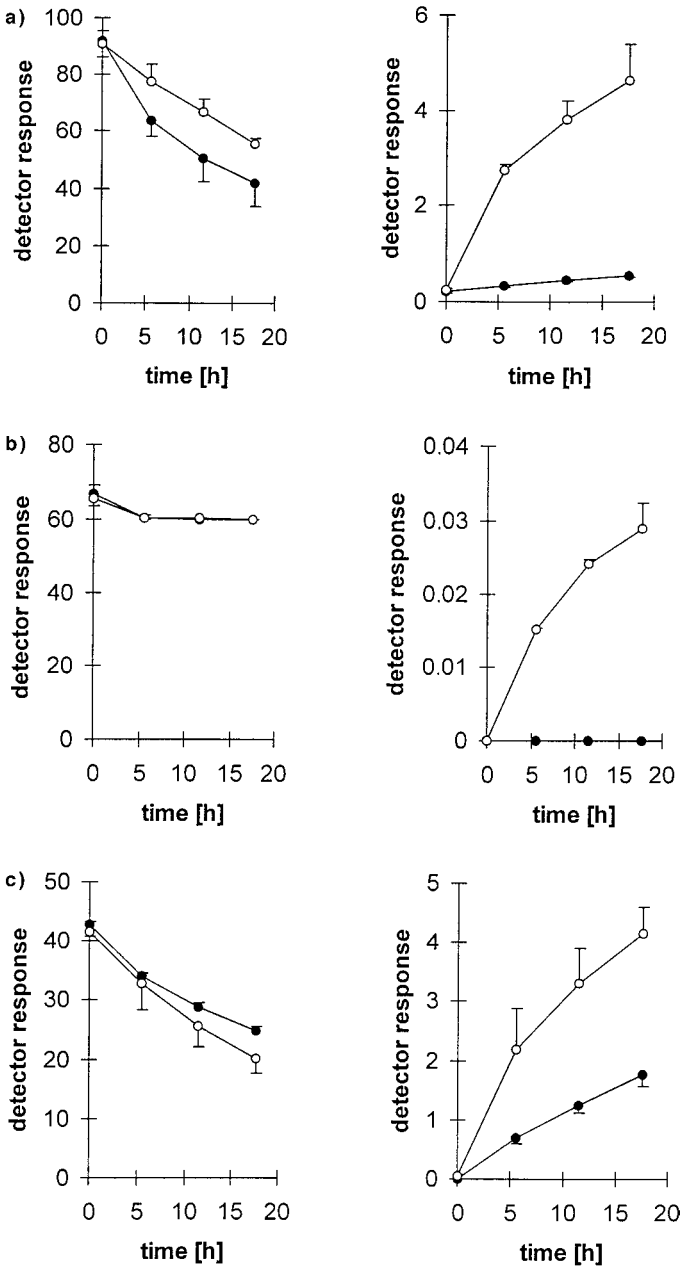

Fig. 2. *Decay* (left-hand) *of* a) *7,8-dihydroneopterin* (**2**), b) *sepiapterin* (**8**), *and* c) *7,8-dihydro-6,7,8-trimethylpterin* (**5**) *and hydroxylation of salicylic acid in the presence* (○) *or absence* (●) *of ferric ions/EDTA, and concomitant formation of 2,5-dihydroxybenzoic acid* (DHBA) (right-hand). Pterins (500 μM) were incubated together with 500 μM salicylic acid in the presence or absence of 20/40 μM ferric ions/EDTA. After the time periods indicated, aliquots were analyzed by HPLC, and dihydropterins and DHBA[1]) were quantified (mean values ± s.d. of 3 runs). Note the different scale in *Fig. 2,b* (right-hand).

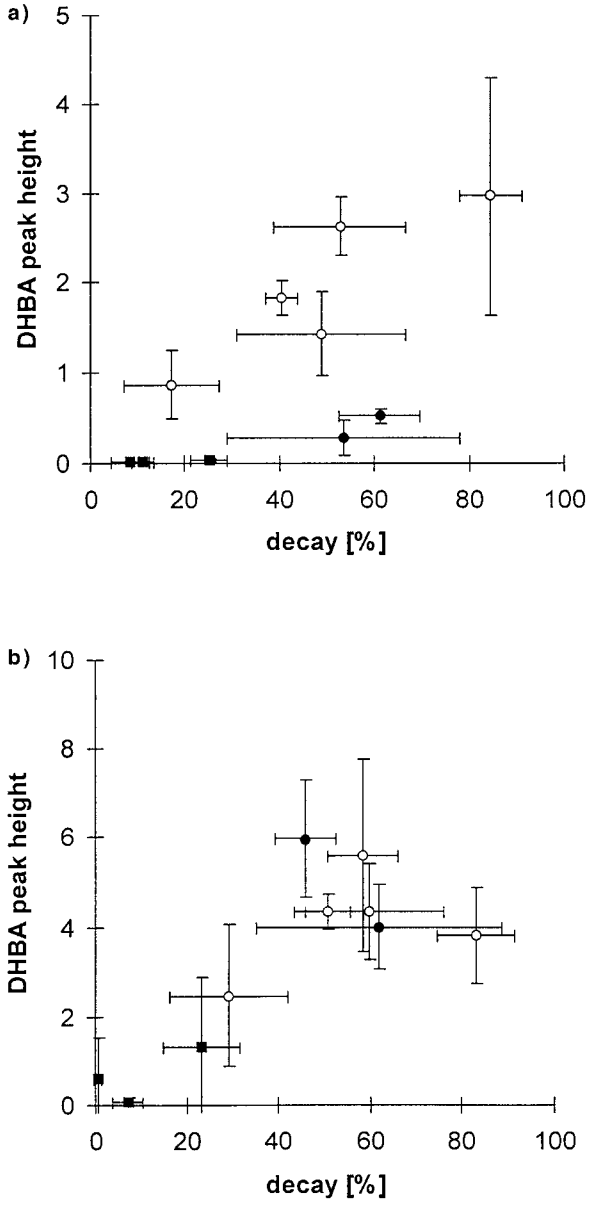

Fig. 3. *Correlation of DHBA formation with dihydropterin decay in the case of 1′-oxo derivatives* **8**–**10** (■); *1′-hydroxy derivatives* **1** *and* **2** (●), *and methyl derivatives* **3**–**7** (○): a) *absence of ferric ions/EDTA and* b) *presence of ferric ions/EDTA*. Dihydropterins (500 μM) were incubated in the presence of 500 μM salicylic acid without or with 10/20 μM Fe^{3+}/EDTA for 17.5 h, and then the amounts of produced DHBA and dihydropterin determined (mean values ± s.d. of 3 runs).

trend, hydroxylation is increased considerably by addition of EDTA-chelated[1]) ferric ions, depending on the group of compound. Therefore, changes in the stability are not responsible for an altered extent of hydroxylation on addition of 10 μM Fe^{3+}. In solutions of the methylated dihydropterins **3**–**7**, only a 1.4 to 3.1 fold increase of hydroxylation is observed on addition of Fe^{3+}/EDTA, while 6-(1′-hydroxy) and 6-(1′-oxo) derivatives **1** and **2** and **8**–**10**, respectively, give at least a tenfold increase. The relative increase is highest with the 6-(1′-oxo) derivatives (up to 65-fold), but only because of the low basal value. In total amounts, 6-(1′-hydroxy) derivatives **1** and **2** show the highest response to the addition of chelated iron. The pattern in the correlation of hydroxylation with the decay of the pterin changes on addition of chelated ferric ions. There are no longer separable groups of different pterin classes, but a single group including all tested compounds showing a good correlation of hydroxylation with pterin decay with $r^2 = 0.626$ (*Fig. 3*,*b*).

From the consecutive oxidation of the analytes on 8 electrodes in series, the hydrodynamic voltammogram of every 7,8-dihydropterin can be derived (*Fig. 4*). The two hydroxy derivatives **1** and **2** as well as the three oxo derivatives **8**–**10** show similar voltammograms and nearly identical potentials for half-maximal oxidation (*Fig. 4*,*a*). On the other hand, methyl derivatives **3**–**7** are found to have oxidation potentials over a broad range (*Fig. 4*,*b*), indicating a more pronounced impact on the oxidation potential by substitution at the pterin-ring system as compared to the impact of different side chains at position 6. Correlation of the oxidation potentials with the amount of hydroxylation gives a pattern comparable to *Fig. 3*,*a:* in the case of methyl derivatives **3**–**7**, hydroxylation is higher for compounds that are easily oxidized (*Fig. 5*). One exception is 3,6,7,7-tetramethyl-7,8-dihydropterin (**7**), which is the only compound methylated at the pyrimidine moiety. Both other dihydropterin groups show a clustering where, again, lower potentials are associated with higher hydroxylation. In the experiments with added ferric ion, no more clustering or correlation could be found.

Within groups of chemically similar dihydropterin derivatives, hydroxyl-radical formation depends on the decay of the dihydropterin, which is in agreement with its oxidation potential. Comparing different dihydropterin groups reveals the same trend: those that are more easily oxidized give higher hydroxylation yields. In the presence of chelated ferric ion, the extent of hydroxylation is well-correlated with dihydropterin decay, including compounds of all groups. The most dramatic effect is observed for solutions of 7,8-dihydroneopterin (**2**) and 6-(hydroxymethyl)-7,8-dihydropterin (**1**), both with a 1′-hydroxy group. While **1** gives only *ca.* 10% hydroxylation compared to its 6-methyl analog **4** without added iron, in the presence of iron both **1** and **4** show about the same hydroxylation amount. These data suggest that the effect might be due to an enhanced binding of iron ions by dihydropterins containing a 6-(1′-hydroxy) function.

In the case of dihydroneopterin **2**, we elucidated the effects of EDTA and ferric ions further. We found that either EDTA or ferric ions alone increase the stability of **2** considerably (*Fig. 6*,*a*). On the other hand, addition of ferric ions to a solution of **2** containing EDTA decreases the stability of **2** compared to EDTA alone. A completely different pattern is observed for the effect on hydroxylation. While EDTA enhances hydroxylation, ferric ions even decrease the amount of DHBA[1]) formed; the highest

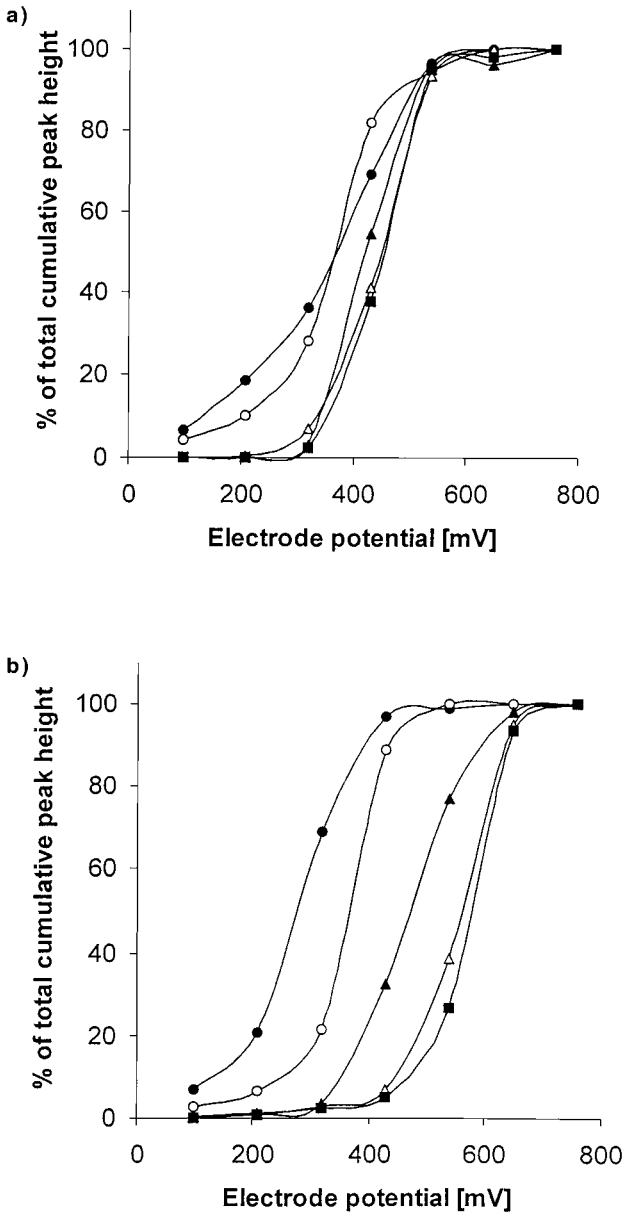

Fig. 4. *Hydrodynamic voltammograms of 7,8-dihydropterins:* a) *7,8-dihydro-6-(hydroxymethyl)-pterin* (**1**; ○) *7,8-dihydroneopterin* (**2**; ●), *sepiapterin*, (**8**; △), *deoxysepiapterin* (**9**; ■) *and 6-formyl-7,8-dihydropterin* (**10**; ▲) b) *7,8-dihydro-6-methylpterin* (**3**; ●), *7,8-dihydro-6,7-dimethylpterin* (**4**; ○), *7,8-dihydro-6,7,7-trimethylpterin* (**6**; △), *7,8-dihydro-6,7,8-trimethylpterin* (**5**; ▲), *and 7,8-dihydro-3,6,7,7-tetramethylpterin* (**7**; ■). After HPLC, the dihydropterins were detected with a coulometric detector containing 8 working electrodes in series, set at 100, 210, 320, 430, 540, 650, 760, and 870 mV, respectively. From the maximum current (peak height) of the first 7 electrodes, the cumulative peak height was calculated for every pterin investigated.

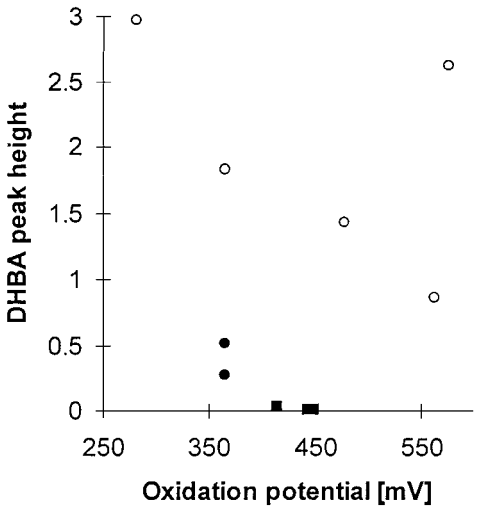

Fig. 5. *Correlation of DHBA formation with dihydropterin oxidation potential: 1′-oxo derivatives* **8**–**10** (■), *1′-hydroxy derivatives* **1** *and* **2** (●), *and methyl derivatives* **3**–**7** (○). The amount of DHBA formed after 17.5 h incubation of 7,8-dihydropterins with salicylic acid without added ferric iron (same values as in *Fig. 3*,*a*) was correlated with the potential necessary for half-maximal oxidation from the hydrodynamic voltammogram.

hydroxylation yields are achieved in the assay containing both EDTA and ferric ions (*Fig. 6*,*b*). The enhancement of hydroxylation by EDTA is attributable to an effective conversion of superoxide to hydroxyl radicals by iron/EDTA. Despite the enhanced hydroxylation, **2** is much more stable in EDTA-containing solution, indicating that **2** does not serve as a scavenger of hydroxyl radicals.

In solutions containing 7,8-dihydroneopterin (**2**) and EDTA with increasing amounts of ferric ions, both the decay rate of **2** and the formation rate of DHBA are increased with increasing iron concentrations. As a consequence, DHBA formation is correlated with the rate constant of dihydroneopterin decay linearily with $r^2 = 0.97$ and a slope of 1.1 (*Fig. 7*). The maximum hydroxylation rate, which can also be calculated from an exponential fit of the amount of DHBA formed *vs.* time, is only slightly influenced by the increase in iron concentration. We have previously shown that the amount of hydroxylation products formed depends on the amount of 7,8-dihydroneopterin (**2**) added [17]. When the dihydropterin is consumed, hydroxylation ceases and starts again after addition of new dihydropterin (*Fig. 8*).

Different dihydropterins of similar structure lead to hydroxylation of salicylic acid in an amount according to the decay of the dihydropterin. The 7,8-dihydroneopterin (**2**), under conditions of differing stability, also induces hydroxylation dependent on its decay. The faster decay of dihydropterins is not attributable to the formation of more hydroxyl radicals, as shown for **2**, which is stabilized by EDTA despite a much higher hydroxylation rate. Hydroxylation is dependent not only on the oxidizability of the dihydropterin used, which is determined primarily by the dihydropterin ring system, but also by different side chains at position 6, especially in the presence of iron ions. This may be due to different iron-chelating properties influenced by different substituents at C(1′).

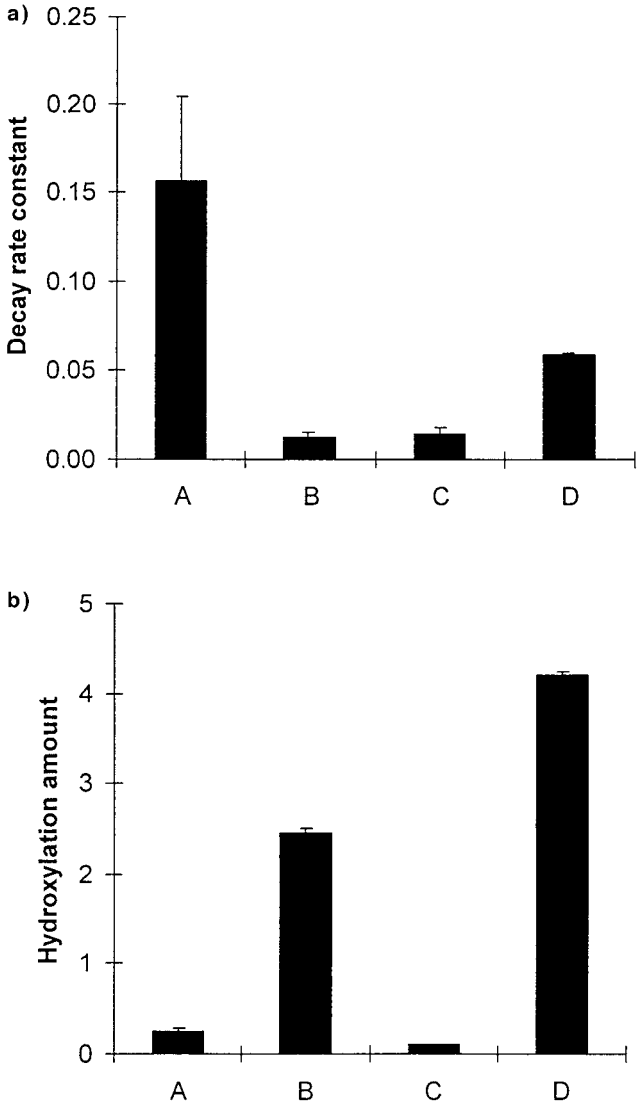

Fig. 6. *Effect of iron and EDTA on the stability of 7,8-dihydroneopterin* (**2**) *and hydroxylation:* a) *decay of* **2** *and* b) *formation of DHBA*. Up to 55 h, **2** (50 μM) was incubated with 500 μM salicylic acid in the absence (A) or presence of 200 μM EDTA (B), 50 μM ferric ions (C) or both (D). After 7 periods of time, the amount of DHBA and **2** in the mixture was measured. From an exponential fit of the decay of **2** and the formation of DHBA, the decay rate constants (*a*)) and formation constants (*b*)) were calculated (mean values ± s.d. of 2 runs).

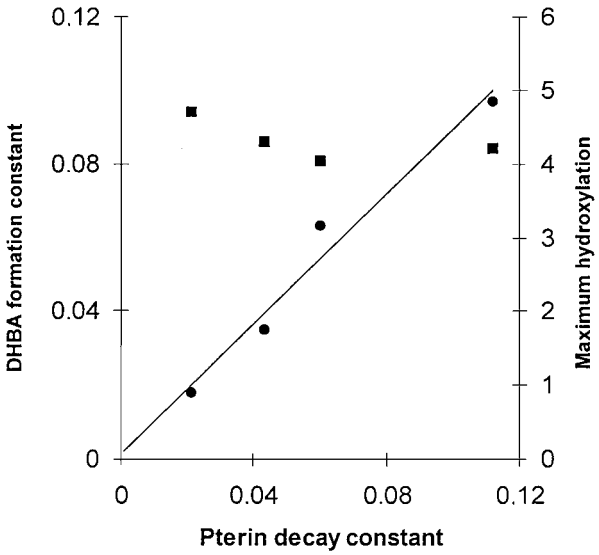

Fig. 7. *Correlation of the DHBA-formation rate constant* (●, left-hand axis) *and maximum hydroxylation* (■, right-hand axis) *in the case of 7,8-dihydroneopterin* (**2**) *decay.* Up to 55 h **2** (50 μM) was incubated with 500 μM salicylic acid in the presence of 200 μM EDTA and 10, 20, 50, and 100 μM ferric ions. From an exponential fit of the decay of **2** and the formation of DHBA, the decay constants, formation constants (●) and maximum hydroxylation (■) were calculated.

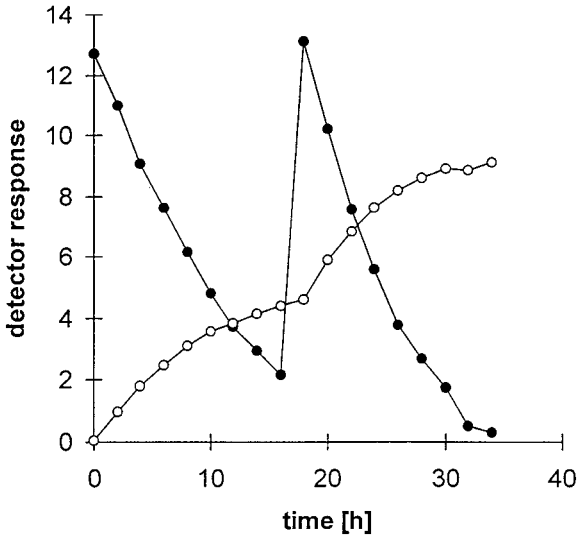

Fig. 8. *The 7,8-dihydroneopterin* (**2**) *as electron donor: amounts of* **2** *and DHBA* vs. *time.* Dihydroneopterin **2** (100 μM) was incubated with 200 μM ferric ions and 400 μM EDTA. After the time periods indicated, aliquots were analyzed and **2** (●) and 2,5-DHBA (○) quantified. After 18 h, the amount of **2** was brought to the initial concentration and the incubation prolonged for a further 16 h.

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Experimental Part

General. 7,8-Dihydro-6-methylpterin (**3**), 7,8-dihydro-6,7,7-trimethylpterin (**6**), 7,8-dihydro-6,7,8-trimethylpterin (**5**), and 7,8-dihydro-3,6,7,7-tetramethylpterin (**7**) were synthesized as described elsewhere [19][20]. All other pterins were obtained from *Dr. Schircks Laboratory* (Jona, Switzerland). The 2,3-dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid (DHBA) were purchased from *Sigma* (Vienna, Austria). Phosphate buffered saline (PBS), consisting of 1.15 g/l of Na_2HPO_4, 0.2 g/l of KH_2PO_4, 0.1 g/l of $CaCl_2$, 0.1 g/l of $MgCl_2 \cdot 6\ H_2O$, 0.2 g/l of KCl, 8 g/l of NaCl, pH 7.4, was obtained from *PAA* (Linz, Austria). All other reagents were obtained from *Merck* (Darmstadt, Germany).

Analyses of Dihydropterins and Hydroxylation Products of Salicylic Acid by HPLC. Hydroxylation assays were carried out in brown 2-ml *Chromacol* autosampler vials with incubation times up to 24 h. After the indicated time intervals, 20-µl aliquots of the mixture were analyzed by HPLC. The system consisted of an *ERC* vacuum degasser (Kawaguchi City, Japan), a *Flux-Rheos-4000* quaternary gradient pump (Basel, Switzerland), two pulse dampeners in series, a *Spark-Triathlon* autosampler (Emmen, Netherlands) with an inert sample needle and a 20-µl sample loop, a *Spark Marathon* column oven and an *ESA-Coularray* multielectrode coulometric detector (Chelmsford, MA, USA), equipped with eight graphite working electrodes. The potentials of the electrodes were set at 100, 210, 320, 430, 540, 650, 760, and 870 mV against a Pd reference electrode. Separation was performed on a *Bischoff* column (150×3 mm) filled with *Waters Spherisorb ODS2* (3 µm particles). Mobile phase was delivered at a flow rate of 0.5 ml/min by a gradient elution of 100% solvent *A* for 5 min, then raising solvent *B* within 10 min to 30%. After 5 min, solvent *A* was raised again to 100% within 5 min. Before the next run, the column was equilibrated for 10 min with 100% solvent *A*. Solvent *A:* 30 mM NaOAc, 30 mM sodium citrate (pH 4.6); solvent *B:* MeOH. Column and electrodes were kept at 35°.

With this HPLC system, the different dihydropterins were separated from their degradation products as well as 2,3- and 2,5-dihydroxybenzoic acid (DHBA) and salicylic acid. DHBA was used as a quantitative probe for hydroxyl-radical formation in all experiments. For the quantitation of each compound, the chromatogram obtained by the dominant channel was used.

The incubation mixtures consisted of 500 µM dihydroneopterin and 500 µM salicylic acid in PBS (pH 7.4), unless otherwise stated. After addition of 500 µM salicylic acid, the pH of the mixture decreased to 7.2. Incubations were carried out at r.t. Stock solns. of 7,8-dihydroneopterin (**2**) and iron salts were prepared immediately before starting the assay.

Solutions of ferric ions were prepared by dissolving $FeSO_4$ in PBS, which autooxidized immediately [21]. For the preparation of the solns., H_2O deionized by a *Millipore MilliQ 185* (Vienna, Austria) was used.

Computations. The kinetics of the formation of hydroxylated products and the decay of dihydropterins were evaluated by fitting simple exponential functions of the form $c(t) = c_{max} \cdot (1 - e^{-kt})$ and $c(t) = c_0 \cdot e^{-kt}$, respectively, to the temporal profiles ($c(t)$ = time-dependent concentration of hydroxylation products or dihydropterins, c_{max} = finally reached concentration of hydroxylation products, c_0 = initial dihydropterin concentration, k = rate constant). Fitting of the parameters c_{max} and k was performed with the program SCIENTIST (*MicroMath*, *Inc.*, Salt Lake City, Utah, USA). Notably, the standard estimation errors of the parameter estimates are provided by this program.

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