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Formation of Oxygen Radicals in Solutions of 7,8-Dihydroneopterin

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Neopterin and 7,8-dihydroneopterin, two compounds which are secreted by activated macrophages, have been shown to interfere with radicals generated by cellular and certain chemical systems. Reduced pterins were reported to scavenge whereas aromatic pterins promoted or reduced radical mediated reactions or had no effect. However, recently it was found that high concentrations of 7,8-dihydroneopterin enhanced luminol dependent chemiluminescence and T-cell apoptosis, suggesting an enhancement of free radical formation. In this study hydroxylation of salicylic acid was used for detection of hydroxyl radicals. It is shown that in solutions of 7,8-dihydroneopterin hydroxyl radicals were formed in the absence of any radical source. The presence of EDTA chelated iron enhanced hydroxyl radical formation. Whereas the addition of iron accelerated the hydroxylation reaction, 7,8-dihydroneopterin was responsible for the amount of hydroxylation products. In the presence of superoxide dismutase or catalase, as well as by helium purging, hydroxylation was inhibited. Our data suggest that in solutions of 7,8-dihydroneopterin superoxide radicals are generated which are converted to hydroxyl radicals by Fenton or Haber-Weiss type reactions. While superoxide might be generated during autoxidation of ferrous iron, dihydroneopterin seems to be involved in regeneration of ferrous iron from the ferric form.

Human macrophages are known to secrete reactive oxygen species as well as neopterin and dihydroneopterin upon stimulation by interferon γ (1). Despite the intense usage of neopterin as an indicator for activation of the cellular immune system (2) the biological significance of neopterin and dihydroneopterin are obscure. During the past decade evidence was growing that pterin derivatives can be involved in phenomena mediated by free radicals. Among those phenomena are effects on chemiluminescence of luminol or other compounds induced by macrophages (3, 4) or simple 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, most basically, electron spin resonance (ESR) experiments using spin traps (13). The consensus of these data is that reduced pterins generally act as scavengers of free radicals. Aromatic pterins were reported either to have no effect, or to enhance, or to reduce radical mediated reactions. All these experiments reported have in common that the pterin derivatives were exposed to free radical sources and the actions of pterins on those preformed radicals were investigated.

On the other hand it is well known that fully reduced pterins such as, e.g., tetrahydrobiopterin are prone to autoxidation in the presence of molecular oxygen (14, 15), and thereby superoxide radicals are generated. Dihydropterins are more stable in comparison to their fully reduced counterparts but are still sensitive to oxygen (16). Often these facts are not considered when experiments with reduced pterins are performed. Only recently it was found that high concentrations (>1 mM) of 7,8-dihydroneopterin may even enhance radical mediated reactions including luminol dependent chemiluminescence (8) and apoptosis (17, 18). Moreover, we have shown that in solutions of dihydroneopterin in the presence of the spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) the formation of the spin adduct with hydroxyl radicals is observed (19). Stimulated by this finding our study was undertaken to explore in detail the nature and origin of the radicals formed spontaneously in oxygen-containing solutions of dihydroneopterin.

Abbreviations used: DFO, deferoxamine mesylate; DHBA, dihydroxybenzoic acid; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DTPA, dithylenetriamine-pentaacetic acid; EDTA, ethylenediamine tetraacetic acid; ESR, electron spin resonance; PBS, phosphate buffered saline.

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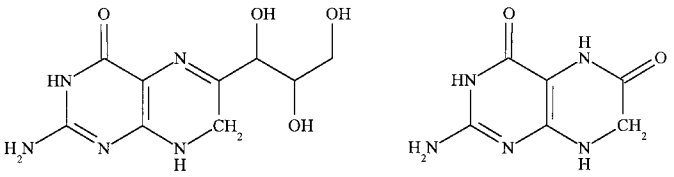

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FIG. 1. Chemical structures of pterins. 7,8-dihydroneopterin (1); 7,8-dihydroxanthopterin (2).

EXPERIMENTAL PROCEDURES

Reagents. 7,8-dihydroneopterin and 7,8-dihydroxanthopterin (Fig. 1) were obtained from Dr. Schircks laboratory (Jona, Switzerland). Superoxide dismutase (EC 1.11.1.6), catalase, (EC 1.15.1.1), deferoxamine mesylate diethylenetriamine-pentaacetic acid (DTPA), 2,3- dihydroxybenzoic acid (2,3-DHBA), 2,5- dihydroxybenzoic acid (2,5-DHBA) and catechol, were purchased from Sigma (Vienna, Austria). Phosphate buffered saline (PBS), consisting of 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.1 g/l $CaCl_2$, 0.1 g/l $MgCl_2.6H_2O$, 0.2 g/l KCl, 8 g/l NaCl, pH 7.4 was obtained by PAA (Linz, Austria). Chelex 100 was from Biorad (Vienna, Austria). All other reagents were obtained from Merck (Darmstadt, Germany).

Analyses of pterins and hydroxylation products of salicylic acid by high performance liquid chromatography. Hydroxylation assays were carried out in brown 2 ml chromacol autosampler vials with incubation times up to 45 hours. At the time points indicated aliquots of 20 μl of the mixture was analyzed by high performance liquid chromatography. 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 300, 380, 460, 540, 620, 700, 780, and 860 mV against a palladium reference electrode, respectively. Separation was performed on a Merck Lichrocart 125 $\times$ 4 mm column with Superspher 100 RP-18 endcapped, 5 μm particles. As mobile phase 30 mM sodium acetate, 30 mM sodium citrate pH 4.6 was used with flow rate of 1 ml/min (20). Column and electrodes were kept at 35°C.

Using this HPLC system we were able to separate dihydroneopterin, its degradation product 7,8-dihydroxanthopterin as well as 2,3- and 2,5-DHBAs, catechol and salicylic acid (Fig. 2). DHBAs could be quantitated at concentrations above 30 nM. We used 2,5-DHBA as a quantitative probe for hydroxyl radical formation in all experiments. For quantitation by an external standardisation the chromatogram obtained by the dominant channel (380 mV) was used.

The incubation mixtures consisted of 500 μM dihydroneopterin, 500 μM salicylic acid in PBS, pH 7.4, unless otherwise stated. After addition of 500 μM salicylic acid pH of the mixture decreased to 7.2. Incubations were carried out at room temperature. Stock solutions of dihydroneopterin and iron salts were prepared immediately before starting the assay.

Solutions of ferric ions were prepared by dissolving $FeSO_4$ in PBS which autoxidizes immediately or by dissolving $FeCl_3$ in PBS (21). When ferrous iron was used $FeSO_4$ was dissolved in water deionized by a Millipore MilliQ 185 (Vienna, Austria) which was continuously degassed by helium.

Computations. The influence of the concentration of 7,8-dihydroneopterin and iron on the kinetics of the formation of hydroxylated products was evaluated by fitting a simple exponential function of the form:

$$c(t) = c_{max} \cdot (1 - e^{-kt})$$

to the temporal profiles. Here; $c(t)$ denotes the time-dependent concentration of hydroxylation products, c_{max} is the finally reached concentration of hydroxylation products and k is the rate constant. Fitting of the parameters $c(t)$ and k was performed using the program SCIENTIST (MicroMath, Inc., Salt Lake City, Utah, USA). Notably, the standard estimation errors of the parameter estimates are provided by this program.

RESULTS

Incubating solutions of 7,8-dihydroneopterin in PBS at pH 7.2 in the presence of salicylic acid we found that 2,3- and 2,5-DHBA as well as catechol are formed (Fig. 2). The formation of these products is time dependent, starts immediately and lasts for several hours (Fig. 3). In contrast, in the absence of 7,8-dihydroneopterin salicylic acid is stable and hydroxylation products were found just as traces. Dihydroneopterin enhanced hydroxyl radical formation in a concentration dependent manner (Fig. 4).

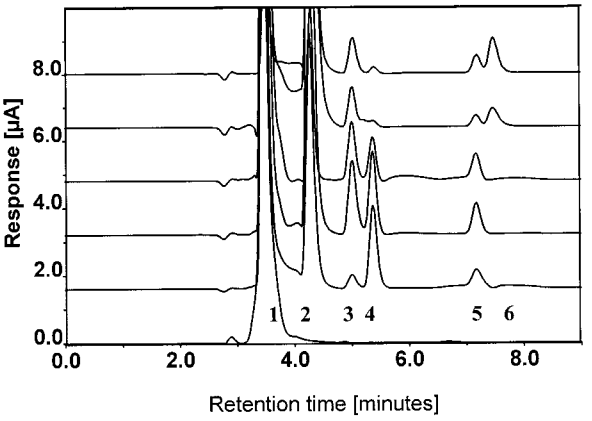

FIG. 2. HPLC analyses of incubations of dihydroneopterin with salicylic acid. Dihydroneopterin (500 μM) was incubated in the presence of 500 μM salicylic acid in PBS, pH 7.2 at room temperature for 24 hours. 20 μl of the assay mixture was analyzed by HPLC. The peaks were identified as dihydroneopterin (1), dihydroxanthoperin (2), 2,3-dihydroxybenzoic acid (3), 2,5-dihydroxybenzoic acid (4), catechol (5), and salicylic acid (6). The chromatograms of six electrodes set at increasing potentials of 300, 380, 460, 540, 620, and 700 mV, respectively, are shown. The main oxidation of salicylic acid happens at potentials above 700 mV which are not shown here.

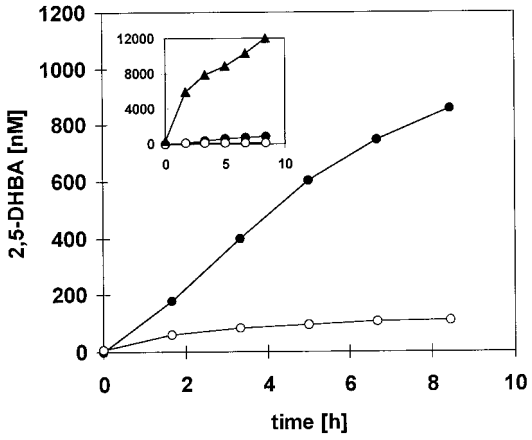

FIG. 3. Time course of formation of dihydroxybenzoic acid. Salicylic acid was incubated in the presence or absence of dihydroneopterin or EDTA chelated ferric iron. After the time indicated 20 μl of the assay mixture were analyzed by HPLC and 2,5-DHBA was quantitated. Salicylic acid + 500 μM dihydroneopterin (●), salicylic acid + 10 μM ferric iron/20 μM EDTA (○), salicylic acid + 500 μM dihydroneopterin + 10 μM ferric iron/20 μM EDTA (▲). The results of a typical experiment are shown.

During the incubation dihydroneopterin was consumed and a new chromatographic peak emerged which was increasing with time. The major reaction product was identified as 7,8-dihydroxanthopterin by its retention time and the hydrodynamic voltammogram compared with the authentic substance.

Addition of EDTA to the assay containing 7,8-dihydroneopterin enhanced the formation of hydroxylation products. On the other hand, addition of DFO instead of EDTA decreased hydroxylation. The treatment of the assay buffer with chelex 100 diminished

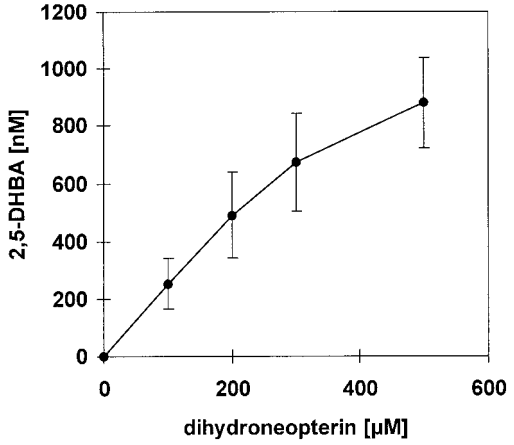

FIG. 4. Concentration dependence of hydroxylation. Salicylic acid was incubated in the presence of increasing concentrations of dihydroneopterin without addition of iron ions for eight hours. The assay mixture was then analyzed by HPLC and 2,5-dihydroxybenzoic acid was quantitated. Mean values ± S.D. of three experiments are shown.

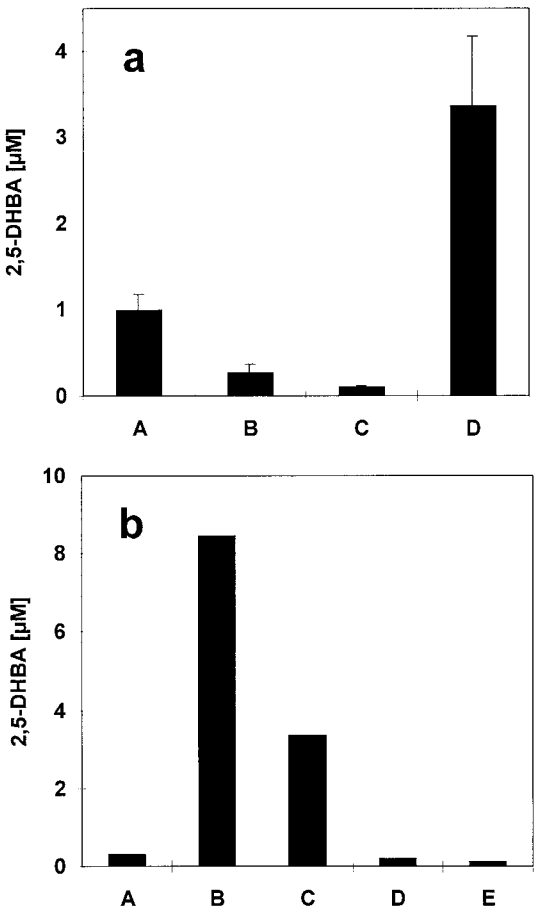

FIG. 5. Effect of chelators and ferric iron on hydroxylation. Salicylic acid was incubated with diyhydroneopterin in the presence or absence of chelators and ferric iron for eight hours. Then the content of the mixture on 2,5-dihydroxybenzoic acid was measured by HPLC. (a) Effect of chelators without addition of iron ions: A, no chelator added; B, assay buffer was treated with chelex-100; C, 20 μM deferroxamine was added; D, 20 μM EDTA was added. Mean values ± S.D. of two experiments are shown. (b) Effect of 10 μM ferric iron in the presence of different chelators: A, 500 μM dihydroneopterin (without iron and chelators); B, ferric iron + 20 μM EDTA; C, ferric iron + 20 μM DTPA; D, ferric iron + 20 μM deferroxamine; E, ferric iron + 20 μM EDTA without dihydroneopterin. The results of a typical experiment are shown.

hydroxylation, too (Fig. 5a). If chelated ferric ions were added to the reaction mixture hydroxylation was increased about twentyfold when EDTA was used, DTPA had half the enhancing effect compared to EDTA, and using DFO hydroxylation was even less compared to the assay with no iron added. In a reaction mixture containing EDTA chelated ferric iron in the absence of 7,8-dihydroneopterin 2,5-DHBA was formed but less compared to 7,8-dihydroneopterin alone (Fig. 5b).

Using high concentrations of ferric ions (50 to 200 μM) hydroxylation ceased after about 24 hours. Exponential curves were fitted to the plot of 2,5-DHBA concentration versus time (Fig. 6). From this fit time constants and maximum concentrations were calculated (Table 1). Dif-

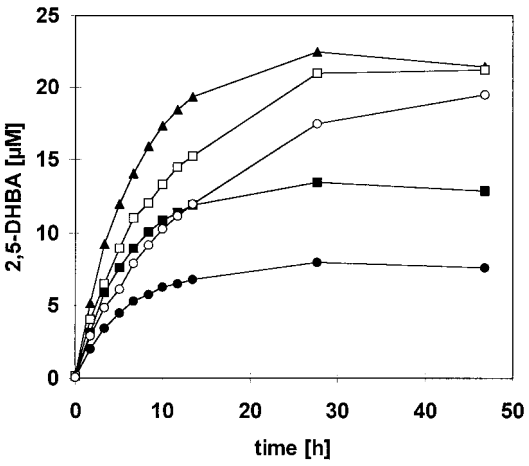

FIG. 6. Kinetic effects of 7,8-dihydroneopterin and chelated ferric ions on hydroxylation. Hydroxylation assays were performed using 500 μM salicylic acid, 400 μM EDTA, and different concentrations of 7,8-dihydroneopterin and ferric ions. At the times indicated 2,5-DHBA concentrations were determined. The concentrations were: 200/200 (▲), 100/200 (■), 50/200 (●), 200/100 (□), and 200/50 (○) μM 7,8-dihydroneopterin/ferric ions, respectively.

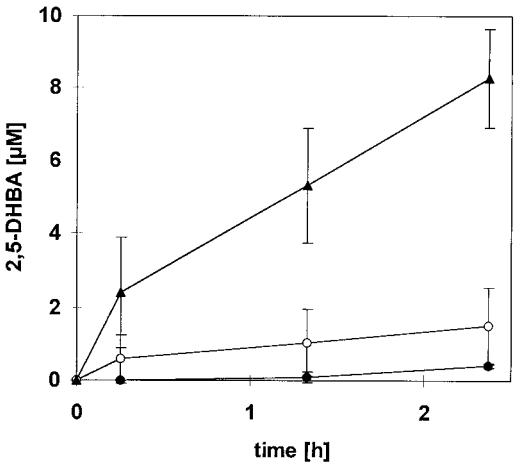

FIG. 7. Effect of ferrous ions on hydroxylation. Salicylic acid was incubated in the presence of 20 μM ferrous ions chelated by 40 μM EDTA (○), 400 μM 7,8-dihydroneopterin (●), or both (▲). At the times indicated 2,5-DHBA was measured.

ferent effects of 7,8-dihydroneopterin and iron ions were found according to the kinetics of hydroxylation. While dihydroneopterin effected primarily the maximum concentration of hydroxylation products and not the time constant, chelated iron in contrast enhanced the reaction rate while the degree of hydroxylation was not effected significantly (Table 1, Fig. 6). In an assay, containing 200 μM EDTA chelated ferric iron 7,8-dihydroneopterin is almost completely depleted after about 24 hours and hydroxylation reaches a plateau. After addition of dihydroneopterin the reaction goes on again with essentially the same characteristics (data not shown).

In contrast to ferric ions ferrous ions chelated by EDTA gave a hydroxylation rate which was higher compared to 7,8-dihydroneopterin alone (Fig. 3 inset, Fig. 7). However, addition of 7,8-dihydroneopterin to ferrous iron enhanced hydroxylation at an extent comparable to ferric iron.

To find out whether superoxide derived from molecular oxygen served as a source of hydroxyl radicals we purged the assay solution with helium to get rid of dissolved oxygen. Thereby the formation of hydroxylation products was reduced to 3.5 ± 4.2% in the absence and 3.9 ± 3.2% in the presence of EDTA chelated ferric iron.

As a more direct evidence for the importance of superoxide in hydroxyl radical formation the effect of SOD was elucidated. By the action of 500 U/ml SOD the formation of 2,5-DHBA in the presence of 7,8-dihydroneopterin and EDTA chelated ferric iron was reduced to 13 ± 11%. Catalase (500 U/ml) reduced 2,5-DHBA formation to 6 ± 5%. When both enzymes were added to the assay mixture hydroxylation was only 1 ± 1% of the uninhibited control reaction.

DISCUSSION

Using ESR spectroscopy with spin trapping it was found that in highly concentrated solutions of dihydroneopterin hydroxyl radicals are formed. For a further elucidation we employed another technique of hydroxyl radical detection for some reasons: the spin adduct of

TABLE I

Different Kinetic Effects of 7,8-Dihydroneopterin and Ferric Ions on Hydroxylation of Salicylic Acid

$FeSO_4$ [μM]	200	200	200	100	50
7,8-Dihydroneopterin [μM]	200	100	50	200	200
c_{max} [μM]	22.00 ± 0.25	13.19 ± 0.13	7.73 ± 0.09	21.59 ± 0.45	20.09 ± 0.37
$k \cdot 10^3$ [min^{-1}]	2.61 ± 0.08	2.86 ± 0.08	2.79 ± 0.09	1.68 ± 0.08	1.20 ± 0.04

Note. Salicylic acid (500 μM) was incubated in the presence of 400 μM EDTA and different concentrations of 7,8-dihydroneopterin and ferric ions. Formation of 2,5-DHBA was followed up to 47 hours. A simple exponential curve was fitted to the graph of 2,5-DHBA concentration versus time. From this curve the maximum concentration of 2,5-DHBA (c_{max}) and the time constant (k) of 2,5-DHBA formation were calculated.

DMPO formed with hydroxyl radicals is not stable, the half life is about 2 to 3 hours (22, 23). In addition to direct formation from hydroxyl radicals DMPO-OH is formed as a degradation product of DMPO-OOH, the spin adduct formed with superoxide which has a half life of a few minutes, depending on the pH (24, 25). ESR measurements cannot be automated like HPLC and so series of analyses are rather laborious in ESR spectroscopy. Aromatic hydroxylation on the other hand is a well established method for the detection of hydroxyl radicals (20, 26, 27). It is specific, the products are stable, automation is possible and the quantitation of the hydroxylation products is easy. Therefore aromatic hydroxylation was the method of choice for us to investigate the formation of hydroxyl radicals.

As we found all three reaction products reported to be formed by the attack of hydroxyl radicals against salicylic acid, we can conclude that indeed hydroxyl radicals are formed in aqueous solutions of dihydroneopterin. That does not necessarily mean that hydroxyl radicals are the original radicals formed.

It has been described that during autoxidation of tetrahydropterins superoxide radicals are generated (15). We speculated that the radical species formed in the presence of 7,8-dihydroneopterin might also be superoxide which is converted to the hydroxyl radical by traces of iron ions in the solution.

The effects of different chelators suggest the involvement of the iron catalysed Haber-Weiss reaction in the formation of hydroxyl radicals. The good Haber-Weiss catalyst EDTA (28–30) promotes hydroxylation, even without addition of iron ions to the assay suggesting that traces of iron contamination contribute to hydroxyl radical formation. On the other hand removement of iron by chelex 100 or addition of DFO which is known to chelate ferric iron and block its reduction to the ferrous form (30, 31) was a good inhibitor of hydroxylation. Iron chelated by DTPA is described to participate in Fenton chemistry but it is suggested that recycling of ferrous iron by superoxide is inhibited by DTPA (32–34). As we found a marked increase in hydroxylation by addition of DTPA chelated iron to our system it seems that 7,8-dihydroneopterin may reduce ferric iron chelated by DTPA.

The inhibition of hydroxylation by purging the assay with helium, addition of SOD and/or catalase support the view that molecular oxygen is reduced via superoxide and hydrogen peroxide to hydroxyl radicals.

The kinetic data clearly show that iron ions are rate limiting but have no influence on the extent of hydroxylation. 7,8-dihydroneopterin serves as the electron donor upon oxidation to 7,8-dihydroxanthopterin and thus is responsible for the amount of hydroxyl radicals formed.

From our data we cannot conclude the mechanism of oxidation of 7,8-dihydroneopterin or whether superoxide is arising from this oxidation or by the autoxidation of ferrous iron. The reaction could be driven directly by the formation of superoxide by 7,8-dihydroneopterin, iron ions being responsible only for the conversion of superoxide to hydroxyl radicals. Ferrous iron would be regenerated by 7,8-dihydroneopterin. Another possibility is the formation of superoxide by the autoxidation of ferrous ion, the role of 7,8-dihydroneopterin being only the reduction of ferric iron to the ferrous form (35).

The enhancement of radical mediated reactions by 7,8-dihydroneopterin described previously was achieved only when concentrations >1 mM have been used (8, 17, 18). Lower concentrations have been reported to scavenge oxygen radicals. In contrast, we demonstrate considerable hydroxylation in the presence even of 50 μM 7,8-dihydroneopterin. Although this is a value far beyond the normal serum concentrations seen during macrophage activation, local concentrations at the site of the activated macrophages might well be in a range for significant enhancement of radical formation.

Our data provide a basis for understanding the role of 7,8-dihydroneopterin as radical scavengers as well as promoters of radical formation. Crucial is not the concentration of the compound used but whether preformed radicals react with the pterin or the pterin acts as a reducing agent in the presence of oxygen and iron ions.

A possible biological role of our findings might be drawn from the fact that 7,8-dihydroneopterin is excreted by activated macrophages simultaneously with the oxidative burst (36). While until now reduced pterins were described to be radical scavengers and thought to be involved in protection against reactive oxygen species, our data show that 7,8-dihydroneopterin can be involved in the generation of oxygen radicals, especially in the presence of iron ions. In this context it is notable that cell mediated oxidation of LDL which is achieved by activated macrophages, is dependent on the presence of transition metals (37).

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