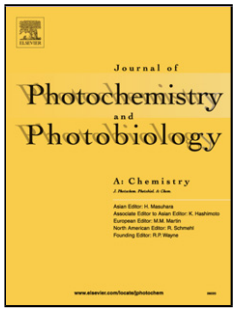

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Transformation of 6-tetrahydrobiopterin in aqueous solutions under UV-irradiation

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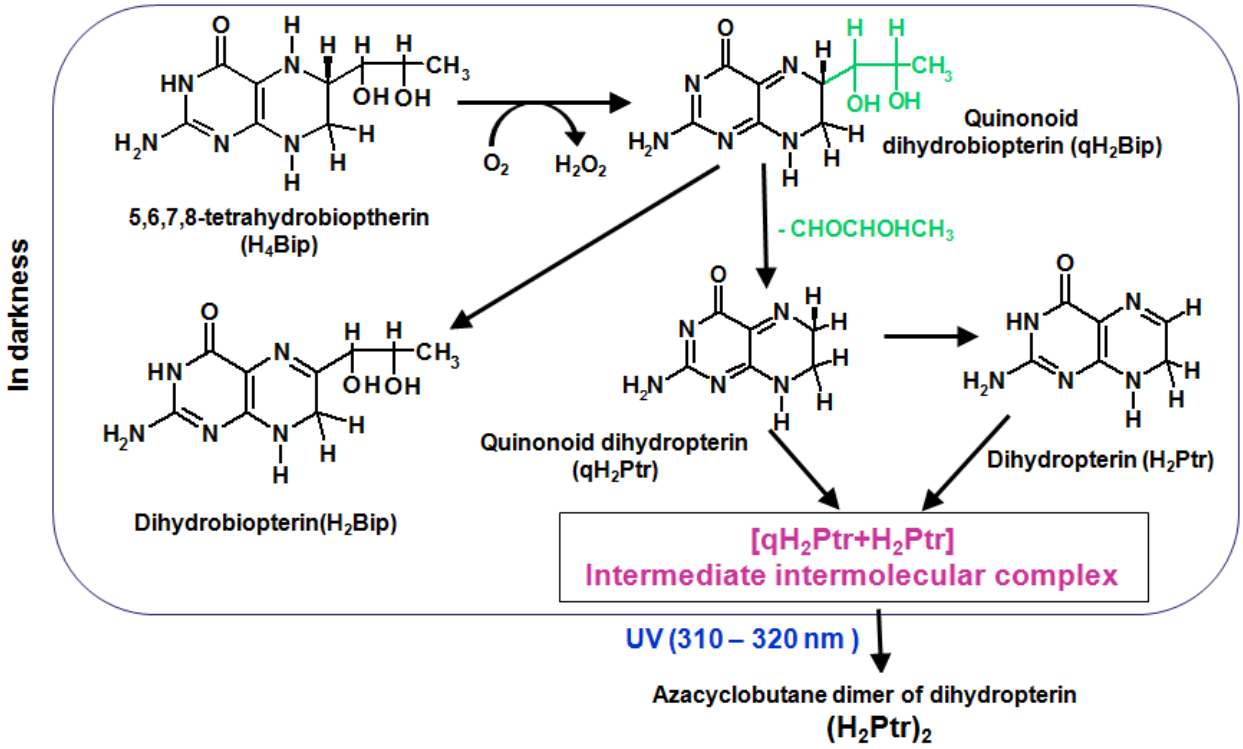
Graphical Abstract



Abstract

Melanogenesis disturbance leads to several pathologies, including vitiligo disease. Ultraviolet (UV) narrowband phototherapy (308 or 311 nm) is used in treating vitiligo; however, the mechanism of phototherapy is not yet understood. Vitiligo is accompanied by three-fivefold increased de-novo synthesis of (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (H_4Bip), its excess and its further oxidation can be considered as significant factors in the pathogenesis of vitiligo. (H_4Bip), as the phenylalanine 4-hydroxylase coenzyme, catalyzes the oxidation of phenylalanine to tyrosine (a melanin precursor). In this context, photo-transformation of H_4Bip in aqueous buffer solutions has been studied. HPLC-MS/MS has demonstrated that pterin products of H_4Bip autoxidation (7,8-dihydropterin (H_2Ptr), dihydroxanthopterin and pterin) predominate over biopterin products (7,8-dihydrobiopterin (H_2Bip) and biopterin). We have shown that UV

irradiation accelerates the autoxidation while the products of oxidative degradation of H_4Bip act as photosensitizers. The distinctive feature of photooxidation of H_4Bip from autoxidation is the formation of dihydropterin $(H_2Ptr)_2$ and dihydrobiopterin $(H_2Bip)_2$ dimers. By means of HPLC-MS/MS it was found that formation of dihydropterin dimers is the predominant process. The signal of molecular ion of the dimer $(H_2Ptr)_2$ (m/z=331) was almost a thousand times higher than the signal of $(H_2Bip)_2$ (m/z=479). The key point of the dimerization is photoexcitation (at 310-320nm) of the intermolecular complex (qH_2Ptr-H_2Ptr) generated in dark. As a result of the photoreaction azacyclobutane dimers have been formed. In the case of alternation of dark and light intervals H_4Bip converted into dimers with 96 % yield. The data obtained are discussed in the context of UV-B narrowband vitiligo phototherapy.

Keywords:
Tetrahydrobiopterin,
Ultraviolet irradiation,
Autoxidation of tetrahydrobiopterin,
Photooxidation of tetrahydrobiopterin,
Azacyclobutane dimer,
Vitiligo phototherapy

1. Introduction

(6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (H_4Bip) is a coenzyme for the series of enzymes: NO synthases, alkylglycerol monooxygenases and aromatic amino acid hydroxylases. H_4Bip is the coenzyme of phenylalanine hydroxylase (EC 1.14.16.1) catalyzing the oxidation of phenylalanine to tyrosine by molecular oxygen in the process of melanogenesis [1]. Melanogenesis disturbance leads is a characteristic feature of certain dermatological pathologies, including vitiligo disease, which results in the emergence of depigmented skin patches. The patients with vitiligo appeared to have three-fivefold increased *de-novo* synthesis of H_4Bip; its excess and its further oxidation can be considered as important factors in the pathogenesis of vitiligo [2,3,4,5,6]. Pterin and its reduced forms absorb light in the ultraviolet spectral region. Recently, tetrahydrocyanopterin has been described as a chromophore within a photoreceptor of several cyanobacteria [7,8]. The role that pterins perform as photoreceptors and as photosensitizers of metabolic reactions is not well understood. It is obvious, however, that these issues are vitally important for photobiology [9] and photomedicine. Apart from our recent work [10], the photochemistry of H_4Bip coenzyme has never been studied. We have demonstrated that biopterin, which has been formed in H_4Bip solution as a result of the dark oxidation, acts as the photosensitizer that accelerates the oxidation of H_4Bip during its exposing to ultraviolet (UV) irradiation [10]. It is known that UV-B irradiation (311 nm), excimer laser (308 nm) and UV-A irradiation (320-400 nm) with photosensitizer (psoralen, etc.) is used in treating vitiligo. It should be mentioned here, however, that the mechanism of therapeutic of 311 nm irradiation is still unknown.

To sum up, the primary goal of the present paper is to study the photo-transformation of tetrahydrobiopterin exposed to UV irradiation in the process of its oxidation by oxygen in water solutions. Another focus is to discuss the obtained results in the context of the mechanism of vitiligo UV therapy.

2. Material and methods

2.1. Chemicals.

Biopterin (Bip), 7,8-dihydrobiopterin (H_2Bip), H_4Bip, 5,6,7,8-tetrahydropterin (H_4Ptr), 7,8-dihydroxanthopterin (H_2Xap) and pterin (Ptr) (purity > 99%) were purchased from Schirks Laboratories, Switzerland, and were used without further purification. Other chemicals used were obtained from Sigma-Aldrich Co.

2.2. Sample Preparation.

Solutions were freshly prepared for each series of experiments by dissolving H_4Bip, H_2Bip, Bip, H_4Ptr, H_2Xap or Ptr in buffer, pH 7.2. Concentration was determined spectrophotometrically using their molar absorption coefficients. Since H_4Bip solutions are very unstable and easily oxidized at neutral pH by atmospheric oxygen, if necessary, aliquots of the samples stabilized by adding 0.25 M Na-citrate buffer pH 2.7 in the ratio 1: 1. We have shown previously that H_4Bip solutions were completely resistant to oxidation by oxygen at pH ≤ 3 [11].

2.3. Experimental methods and apparatus

2.3.1. Steady-state irradiation.

The sets of steady-state experiments were performed to monitor the changes in the electronic spectra of H_4Bip aqueous solutions (pH 7.2) under UV irradiation or without UV irradiation to study autoxidation. The freshly prepared solutions were irradiated under air and constant stirring in 1 cm quartz cell using: (1) DRK 120 high-pressure mercury-vapor lamp (Russia) in combination with UFS-6 light filter (transmission range 300–380 nm) the irradiance was 63 $mWcm^{-2}$; (2) RF-5301 spectrofluorometer Shimadzu (Japan) with Xenon lamp was used as source of monochromatic light at the wavelengths of 300 nm (irradiance 6.9±0.2 $mWcm^{-2}$), 320 nm (irradiance 11.5±0.2 $mWcm^{-2}$) and 350 nm (irradiance 26.0±0.2 $mWcm^{-2}$) with spectral bandwidth of 20 nm. Irradiance value was determined by radiometer Argus-04 (Russia) and Hamamatsu C9386 radiometer (Japan). The first spectrum was measured without irradiation and the following spectra were recorded immediately after exposure.

2.3.2. UV/vis spectroscopy.

Absorption spectra were recorded using spectrophotometers SF-2000 Spektr (Russia), Cary 300 Bio Varian (USA) and Specord 50 (Germany).

2.3.3. High-performance liquid chromatography.

A high-performance liquid chromatographic (HPLC) gradient systems from Stayer, Aquilon (Russia) and Agilent-1200 (USA) were used. The eluate from the HPLC column from Stayer system passed through three detectors connected in series: (1) Stayer UVV-104 UV/Vis detector, Aquilon (Russia); (2) Fluorat-02 fluorimetric detector, Lumex (Russia) that measured fluorescence with excitation at λ= 330 nm and emission at λ= 440 nm, and (3) Tsvet-Yauza-01 amperometric detector, Chimavtomatika (Russia) with working electrode potential of +700 mV. Agilent chromatograph also had spectrophotometeric and fluorimetric detectors.

Fractionation was performed using Partisil SCX and Synergi 4u Polar-RP 80A (250x4.6 mm) columns, Phenomenex (USA). The mobile phases were Ar-saturated.

2.3.4. Liquid chromatography / electrospray ionization mass spectrometry.

HPLC/mass spectrometry (HPLC/MS) was performed by HPLC-ESI-qTof method, using HPLC system (Agilent-1260 Infinity (USA)) consisting of binary pump, thermostat, photometric detector VWD, and the mass spectrometer Agilent 6520 Accurate-Mass quadrupole time-of-flight (Q-TOF) (USA).

2.4. Quantum-chemical calculations.

Quantum-chemical calculations were performed using Orca 3.0.2 program package [12]. Geometry optimization of H_2Ptr dimers and hessian calculation were performed using the density functional theory method (DFT) and the hybrid functional B3LYP [13,14] with a 6-31G(d,p) basis set. The effect of the solvent was taken into account using COSMO continuum solvation model [15,16] with a dielectric constant for water (?? = 80).

3. Results and discussion

3.1. Autoxidation of tetrahydropterins.

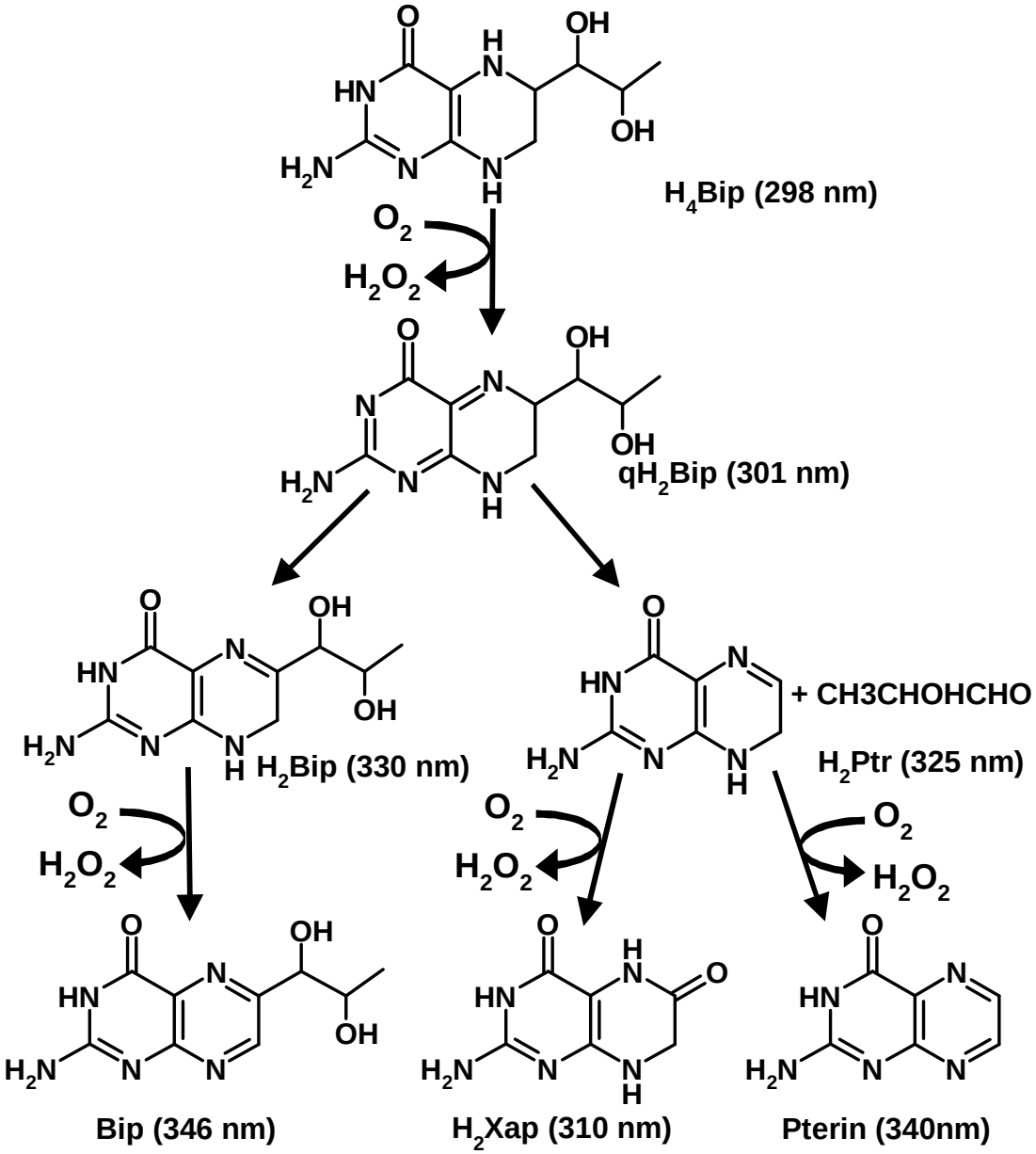
Both *in vivo* and in aqueous solution *in vitro*, H_4Bip is subjected to autoxidation, i.e. oxidation by molecular oxygen [17]. The process of autoxidation is initiated by spontaneous reaction: electron transfers from H_4Bip to oxygen molecule, which results in the formation of superoxide anion radical $O_2^{\bullet-}$ and tetrahydrobiopterin cation radical $H_4Bip^{\bullet+}$ [18]:

$$H_4Bip + O_2 \rightarrow H_4Bip^{\bullet+} + O_2^{\bullet-} \qquad (1$$

or in the formation of trihydrobiopterin radical $H_3Bip^{\bullet}$ and hydroperoxyl radical $HOO^{\bullet}$ [19]:

$$H_4Bip + O_2 \rightarrow HOO^{\bullet} + H_3Bip^{\bullet} \qquad (2)$$

Free radicals and reactive oxygen species that participate in further reactions activating molecular oxygen and oxidizing H_4Bip. In general, the entire process of autoxidation has a radical chain character [19, 20]. The first molecular product of H_4Bip autoxidation is quinonoid 6,7-dihydrobiopterin (qH_2Bip) with the halftime ($t_{½}$) about 1.5 minutes [17]. Then qH_2Bip isomerizes to more stable H_2Bip or is transformed into 7,8-dihydropterin (H_2Ptr) by side-chain cleavage. H_2Ptr is subsequently oxidized by O_2 to H_2Xap [17] and to pterin. Then H_2Bip may be oxidized to Bip [17] (Scheme 1).



Scheme 1. Scheme of tetrahydrobiopterin autoxidation pathway. Within the brackets: the long-wave maximuma of absorption spectra

Temperature, nature and pH of buffer influence the rearrangement qH_2Bip up to a certain dihydroform. Armarego et al. [21] have stated that while H_4Bip is being autoxidized, H_2Ptr tends to prevail in tris-buffer (pH 7.6). In addition, according to Davis et al. [17], in tris-buffer (pH 6.8) H_2Ptr as well tends to prevail considerably; while in phosphate buffer (pH 6.8) it is H_2Bip which prevails. The rise of temperature from 0^oC to 50^oC promotes eliminating of the side radical from qH_2Bip and accumulating of H_2Ptr in 0.1 M phosphate buffer both with pH 6.8 and with pH 7.6. We have studied the kinetics of the initial stage of autoxidation H_4Bip and H_4Ptr with the concentration 1.5×10^{-4}M in tris-buffer and in phosphate buffer (pH 7.2). The products of oxidation in tris-buffer and in the phosphate buffer were analysed by means of HPLC method with photometric and fluorimetric detectors on

cation-exchange column Particil SCX (pH 2.7) in the manner that has already been described [10]. Chromatographic analysis with fluorimetric detection has showed that H_2Ptr and H_2Bip are formed simultaneously since the very beginning of H_4Bip autoxidation. In order to find out the way buffer characteristics affect the elimination of dihydroxypropyl radical from qH_2Bip, the kinetics of accumulating H_2Bip and H_2Ptr have been studied. Fluorimetric detection of H_2Bip and H_2Ptr made it possible to compare the constants of the initial rates at which these dihydropteridins were formed. Thus, the initial rate of H_2Ptr formation (according to the results of fluorescence) in tris-buffer tends to threefold exceed the rate of H_2Bip formation (the constants of rate are equal to 8.5×10^{-4} sec^{-1} and 2.9×10^{-4} sec^{-1}, respectively). Rate constants were evaluated as the rate constants of the first-order reactions. In phosphate buffer the initial rate of H_2Ptr formation also exceeded the rate of H_2Bip formation. The constants of the rates of H_2Bip formation in phosphate buffer and tris-buffer are equal to 1.6×10^{-4} sec^{-1} and 2.9×10^{-4} sec^{-1}, respectively.

We suppose that fluorescence detection results do not show the actual relation of pterin and biopterin products, because H_2Xap (as an example) does not fluoresce and, thus, does not contribute to the total sum of the oxidized pterin derivatives. That is why we use HPLC/MS to identify the products of the H_4Bip autoxidation. The oxidation of H_4Bip (1.5×10^{-4}M) was performed in three 0.1M buffers (ammonium-acetate buffer, phosphate buffer, and tris-buffer) with pH 7.2. The products of the oxidation were divided on Synergy Polar column (0.05M ammonium formate, pH 6.2, 0-60% acetonitrile gradient) and further by mass-spectrometry (MS/MS) were identified. Table 1 presents results of mass-spectrometry analysis.

Table 1.
The effect of the type of buffer on the product distribution from the autooxidation of H_4Bip at 25°C, pH 7.2, 60 min.

	Component in the sum of products, (%) (The average of three experiments)				
	H_4Bip derivatives		H_4Ptr derivatives		
Buffer	H_2Bip	Bip	H_2Ptr	H_2Xap	Ptr
Tris	27.6	0.8	67.8	1.7	2.1
Phosphate	34.6	3.7	44.1	11.3	6.0
Ammonium-acetate	15.7	24.2	35.3	7.6	17.2

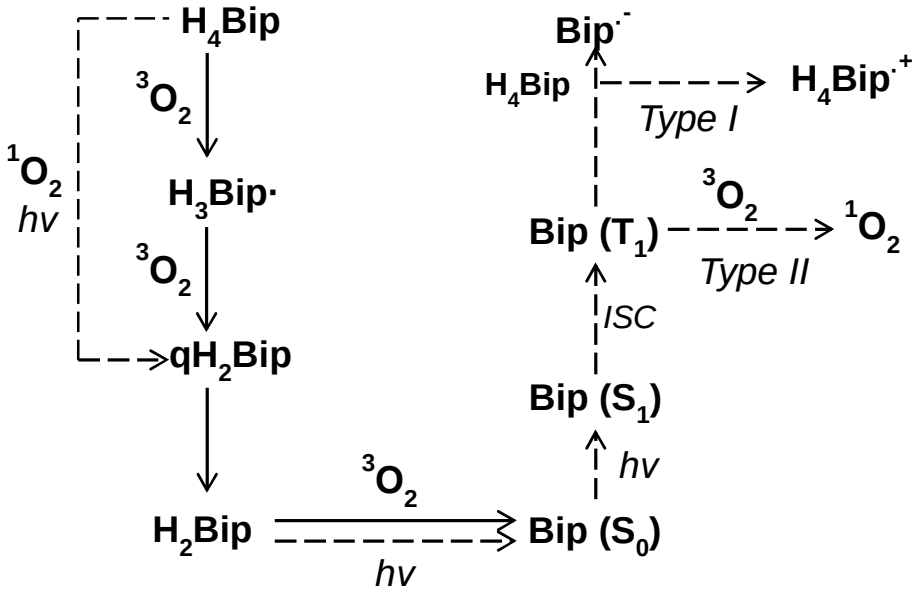
It is clear from the table that in each of three buffers (pH 7.2) the prevailing type of H_4Bip oxidation was the one accompanied by the elimination of the side

radical. Hence, pH can be considered as the one of the main factors that determines the process of H_4Bip autoxidation. In acid medium (pH 3) H_4Bip is completely resistant to the oxidation by oxygen; as pH is shifted to either neutral or alkaline medium, its resistance to oxygen is decreasing. The rate of the quinonoid rearrangement depends on the structure of the buffer anion that interacts with the quinonoid and shifts its electron density. Thus, in tris-buffer ratio of the sum (in %) of the pterin oxidized products (H_2Ptr + H_2Xap + Ptr) to the sum of the biopterin oxidized products (H_2Bip + Bip) is equal to 71.6/28.4; in phosphate buffer – 61.7/38.3; in ammonium-acetate buffer – 60.1/39.9.

To conclude, the autoxidation of H_4Bip in neutral water solutions leads to the formation of biopterin derivatives (H_2Bip and Bip) and, simultaneously, oxidized pterin derivatives (H_2Ptr, Ptr and H_2Xap) are formed.

3.2. Acceleration of tetrahydrobiopterin autoxidation by UV radiation.

Light action directly on H_4Bip (λ_{max} = 298 nm) accelerates the reaction with O_2 and, consequently, accelerates H_4Bip autoxidation. We have shown that UV irradiation of H_4Bip affected its oxidation rate (quantum yield $\Phi_{300} = (2.7\pm0.4) \times 10^{-3}$). The effect of UV irradiation at λ = 350 nm on H_4Bip oxidation was found to be stronger ($\Phi_{350} = (7.7 \pm 1.2) \times 10^{-3}$), especially in the presence of Bip ($\Phi_{350} = (9.7\pm1.5) \times 10^{-3}$) [10]. The rate of H_4Bip oxidation linearly depends on Bip concentration. Experiments with KI, a selective quencher of triplet pterins at micromolar concentrations, have demonstrated that the oxidation is sensitized by the triplet state biopterin ^{3}Bip. Apparently, electron transfer sensitization (Type-I mechanism) is dominant. Energy transfer (Type-II mechanism) and singlet oxygen generation play only a secondary role (Scheme 2). Bip is formed as a result of both autoxidation and photooxidation of H_4Bip. The mechanisms of these photoprocesses are described in detail in our article [10]. The process of photosensitized H_4Bip oxidation is induced by Bip (obviously, other oxidized photosensitizing pterins besides Bip, such as 6-carboxypterin, 6-formylpterin and pterin, can photoinduce H_4Bip oxidation) [22,23,24].



Scheme 2. Proposed tetrahydrobiopterin oxidation pathways under UV-irradiation. Reactions that occur under UV irradiation are shown by dotted arrows; reactions that occur without UV are shown by solid arrows.

We believe that our data may help to shed light on the pathological processes occurring during vitiligo, because the processes described in this article may occur in vivo and lead to the progression of vitiligo pathology.

3.3. The photodimerization of dihydropterins

The effect of photoexcitation on the oxidation of H_4Bip was investigated by means of irradiation of its solutions (pH 7.2) with UV light (300 - 380 nm) using (1) and (2) light sources. It was found that the products of H_4Bip (Fig. 1b) photooxidation differ from the products of autoxidation (Fig. 1a). Spectral analysis of the photooxidation process has shown that in this case absorption band that is typical of H_2Bip does not appear, while this band arises at autoxidation (Fig. 1a). During the photooxidation process, the increase of absorption at 240-250 nm was observed.

HPLC analysis of the photooxidation products (Fig. 2) has revealed that among the products a considerable amount of a new compound occurs, while it is not present among the products of dark oxidation. It should be noted that the process of accumulating the new compound (Fig. 2b) and the increase in absorption at 240-250 nm (Fig. 1b) unfold simultaneously. A difference spectrum (Fig. 1b, inset) reflects the accumulation of product with the maximum absorption at 245 nm and an arm at 275 nm. The spectral characteristics of the compound under discussion are close to dimers of H_2Bip (the maximum absorption on 246 nm and an arm on 280 nm), a small amount of which is formed by photoexcitation of H_2Bip performed without oxygen [25,26]. It should be specially mentioned here that 280 nm corresponds with the maximum point of the penultimate band in the absorption

spectrum of H_2Bip, while for H_2Ptr the maximum point is at 275 nm. A question arises, what are physicochemical characteristics of new compound? This compound does not fluoresce; in absorption spectra it has an unclearly expressed band at 275 nm. During the chromatography on cation-exchange column Particil with pH 2.7 (Fig. 2b), its retention time is considerably longer than that of the other photooxidation products which have the similar structure (H_2Bip, H_2Ptr, Bip, Ptr). Consequently, there is a large positive charge on the compound. This may be conditioned by protonation in acidic medium of two pterin structures at once within one compound. Taking into account the identified physicochemical characteristics, we supposed that the dimers of dihydropterin were formed as a result of photooxidation of H_4Bip. We studied action spectrum of dimerization. It was ascertained that the maximum of the action spectrum is at 310-320 nm.

There is an experimental evidence to support our assumption that it is predominantly photoexcited molecules of dihydropterin, which tend to dimerize. To prove the point H_4Bip and H_4Ptr were irradiated at equal concentrations with monochromatic light at 320 nm. The total exposure time was 20 minutes for H_4Bip and 16 minutes for H_4Ptr (Fig. 3). After every two minutes of exposure the absorption spectra were recorded. At that, dark intervals lasted for 2 minutes as well. As a result, the absorption at 298 nm, which is typical for tetrahydroforms of pterins, decreased for both H_4Bip and H_4Ptr, at the same time the absorption at 240-250 and 275 nm increased. The rate of dimer formation during H_4Bip oxidation (2.0×10^{-7} M sec^{-1}) was lower than that at H_4Ptr oxidation (3.5×10^{-7} M sec^{-1}). The difference in the rates of dimer formation probably derives from an amount of time taken to eliminate the side chain from C6 atom of qH_2Bip.

Differences in the reaction rate of dimerization can also be explained by steric hindrance in the case of H_4Bip. During the cycloaddition reaction, side substituent can prevent overlapping of the frontier orbitals of the two reactants.

Chromatography of the products of H_4Bip and H_4Ptr photooxidation on the Synergy Polar column (0.05 M ammonium acetate, pH 7.0, 0-40% acetonitrile gradient) showed that the main formed dimer was the dimer of dihydropterin $(H_2Ptr)_2$ with retention time 3.8 min (Fig. 4) This finding confirmed that H_2Ptr takes part in forming dimers during H_4Bip photooxidation.

HPLC-MS/MS also proved that irradiation of H_4Bip causes predominantly forming the dimers of dihydropterin. On a Synergi Polar column (0.05M ammonium formate, pH 6.2, 0 - 60% acetonitrile gradient, the length of registered wave was 245 nm) we managed to divide the dimers of dihydropterin and the dimers of dihydrobiopterin. The mass spectrum of the photooxidation products contained the

dominant signal MH^+/z = 331, that corresponds to mass of the dimer $(H_2Ptr)_2$ – 330 Da, and the small signal MH^+/z = 479, that corresponds to mass of the dimer $(H_2Bip)_2$ – 478 Da. The signal of molecular ion of the dimer $(H_2Ptr)_2$ was almost a thousand times higher than the signal of $(H_2Bip)_2$. It worth mentioning here that the dimers $(H_2Ptr)_2$ remained intact after they had being kept in the fridge for 24 hours (~ +6°C), while the dimers $(H_2Bip)_2$ – disappeared.

When the total exposure time was 20 minutes and there was an alternation of dark intervals (1.5 min) and light intervals (0.5 min), the yield of dimers (on the initial tetrahydropterins in calculation) reached 96 % from H_4Bip and 100 % from H_4Ptr. When irradiation process was not interrupted for 20 minutes this values were equal to 64 % and 76 %, respectively. Therefore, in the case of alternation of dark and light intervals H_4Bip converted into dimers almost quantitatively. It is possible that in dark the formation of an intermediate intermolecular complex (qH_2Ptr-H_2Ptr), in which isomerization of quinonoid to 5,8-dihydropterin and further photodimerization of dihydropterins, will take place. The key point of the dimerization is photoexcitation of the intermolecular complexes (qH_2Ptr-H_2Ptr) generated in dark. Hence, it is important to alternate dark and light intervals. We can assume that dark interval must be equal to or exceed the halftime ($t_{1/2}$) of qH_2Bip (1.5 min).

Data on irradiation of H_2Bip support our assumption that intermediate molecular complexes (qH_2Ptr-H_2Ptr) take part in the process of photodimerization. UV (300-380 nm or 320 nm) irradiation of H_2Bip in the presence of oxygen did not cause the formation of dimers. In this case, only the oxidation took place and Bip and H_2Xap were formed. We observed the dynamics of forming the two latter compounds by means of evolving UV absorption spectra, HPLC and HPLC/MS. It should be mentioned here that at the initial stage of irradiation an insignificant amount of dimers, probably, was formed (difference absorption spectra of the first minute of irradiation did prove this fact). This seems to be connected with a possibility of occurring disproportionation reaction $H_2Bip \rightarrow H_4Bip + Bip$. Then a small amount of dimers was formed from H_4Bip. Meanwhile, in general, the oxidation of H_2Bip to Bip and H_2Xap and to a series of other oxidized derivatives was the main point. From this, we can conclude that the intermediate quinonoid dihydro form of pterin is the necessary participant in the process leading to dimers.

The possible formation of intermolecular donor-acceptor complexes between H_2Ptr and qH_2Ptr derives from the following statements:

(1) Between two tautomeric pterin forms created by means of lactam-lactim tautomerism (N=C-OH ↔ NH-C=O), at pH 7 the lactam form prevails; but the lactim form has more nucleophilic charachteristics and, thus, it should be more

effective in the formation of complex with electrophilic quinonoid. UV irradiation may promote the formation of such complexes, since the lactim form seems to be more active under irradiation [27].

(2) By means of circular dichroism (CD), it was demonstrated that intermediate intermolecular complexes (qH_2Ptr-H_2Ptr) appear to participate in photodimerization of dihydropterins. The absorption spectrum of H_4Bip in the 200-250 nm region has a brightly pronounced band with maximum of 221 nm (Fig. 5b); in the CD spectrum this maximum is absent but a positive band at 228 nm and a negative band with a maximum of about 205 are observed (Fig.5a). Apparently, the absorption band at 221nm is a superposition of these two bands. As H_4Bip photooxidation process proceeds, both bands in the CD spectrum disappear synchronously. On the photoformation of dimers can be judged by the appearance of absorption bands of dimers at 243 and 275 nm in the difference absorption spectrum (Fig. 5b, inset). The dimers with an absorption maximum of 243 nm do not give significant CD signals, that is, they have a very weak optical activity. Intermolecular complexes (qH_2Ptr-H_2Ptr) formed as an intermediate product do not contribute to CD signals, since they do not possess optical activity. This is due to the fact that the chiral center is present in qH_2Bip, but in qH_2Ptr it is absent. We believe that dihydropterin dimers are mainly formed.

(3) After H_4Bip solution was being irradiated, the effective quantum yield of dimers formation was 0.8. This is evidence that within the intermolecular complex two dihydropterin compounds are optimally space oriented for photoreaction of dimerization.

As the formation of intermolecular complex is a biomolecular reaction, the formation of the intermediate complex seems to be the limiting stage of the whole process. Substituents that are located near the reaction center usually affect the kinetics of biomolecular reaction. In our case, the side C6-radical in H_2Bip and qH_2Bip may probably act as such the substituent. This fact might be one of the reasons why these are mainly the dimers of dihydropterin $(H_2Ptr)_2$ that are formed during the H_4Bip photooxidation.

Based on B3LYP/6-31G(d,p) method we compared Gibbs free energies of the azacyclobutane H_2Ptr dimers. We also compared Gibbs free energies of cyclobutane dimers proposed by M. Vignoni and co-authors [25,26]. Azacyclobutane dimer (*cis* isomer) is > 230 kJ mol^{-1} more stable than the most stable cyclobutane dimer (anti (h,t) isomer) (Fig. 6) Also, the *cis*-form of the azacyclobutane dimer is 1.6 kJ mol^{-1} more stable than the *trans*-form. Although the *cis* and the *trans*-form population is almost equal (65% and 35%, respectively, according to Boltzmann distribution), we conclude that the most stable H_2Ptr dimer is the azacyclobutane *cis* isomer.

4. Conclusion

In dark H_4Bip is easily oxidized by molecular oxygen (autoxidation) to H_2Bip and Bip. Simultaneously, elimination of the side-chain radical to form pterin derivatives (H_2Ptr, H_2Xap, Ptr) occurs. Similar processes can obviously occur during the vitiligo disease and a similar set of products was found in vitiligo-depigmented skin patches. It was shown by MS/MS mass-spectrometry that at pH 7.2 oxidized pterin derivatives are predominant among oxidation products in aqueous buffer solutions.

We showed that under UV irradiation light-induced processes took place in parallel with constantly flowing dark processes. UV accelerated the process of autoxidation by direct excitation of H_4Bip or by excitation of photosensitizers. Products of the dark and light-dependent degradation of H_4Bip (Bip, Ptr, 6-carboxypterin, 6-formylpterin) can act as the photosensitizers [10,23]. We suppose, *in vivo* similar radical processes can lead to the progression of vitiligo pathology.

The main distinguishing characteristic photoreaction of H_4Bip under UV irradiation was the formation of H_2Ptr dimers. Apparently, the dimerization occurs more readily with H_2Ptr than with H_2Bip. In the case of alternation of dark and light intervals, H_4Bip converted into dimers almost quantitatively. The highly effective quantum yield 0.8 of dimers formation is obviously associated with the fact that the photodimerization occurs in the excited intermediate intermolecular complex (qH_2Ptr-H_2Ptr) generated in dark. According to our theoretical calculations, azacyclobutane dimers $(H_2Ptr)_2$ are predominant over cyclobutane dimers, while the *cis*-form of azacyclobutane dimers is the most stable one.

We believe that such formation of dihydropterin dimers may occur *in vivo* during the narrowband phototherapy (308 or 311 nm) of vitiligo patients. It is known that excess of H_4Bip and its oxidation products inhibit melanin biosynthesis. A considerable number of H_4Bip oxidation reactions leads to accumulation of hydrogen peroxide (H_2O_2) and to oxidative stress in vitiligo [2,3,4,5,28,29]. Furthermore, H_2O_2 stimulates biosynthesis of excessive H_4Bip through the induction of GTF-cyclohydrolase I [30]. Thus, we have a ‘vicious circle’ here, which includes excessive H_4Bip. This ‘vicious circle’ can be broken by the reaction of photodimerization, which excludes the H_4Bip excess.

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Captions to figures:

Fig. 1. Evolution of the absorption spectra of H_4Bip in air-equilibrated 0.1 M phosphate buffer (pH 7.2) in the course of time. (a) Autoxidation and (b) Photooxidation under UV irradiation (300-380 nm, 63 mW cm^{-2}). Arrows indicate the changes observed at different wavelengths. $[H_4Bip]_0 = 1.5 \times 10^{-4}$M. Insets show difference spectra (20 min – 0 min).

Fig. 2. HPLC analysis of the products (a) autoxidation and (b) photooxidation of H_4Bip. Partisil SCX ion exchange column. The mobile phase was Ar-saturated 25 mM sodium citrate buffer, pH 2.7, flow rate 0.7 mL min^{-1}.

Fig. 3. Changes in the absorption spectra monitored upon monochromatic irradiation at 320 nm (11.5 mW cm^{-2}) of (a) H_4Ptr (1.75×10^{-4}M) and (b) H_4Bip (1.77×10^{-4}M) in 0.1 M phosphate buffer, pH 7.2. Arrows indicate the changes observed at different wavelengths. Insets show the difference spectrum between the last spectrum (16 or 20 min, accordingly) and the initial one (0 min).

Fig. 4. HPLC analysis of the photooxidation products of (a) H_4Bip (1.5×10^{-4}M) and (b) H_4Ptr (1.5×10^{-4}M). Synergi 4u Polar-RP 80A column. The mobile phase was 50 mM ammonium-acetate buffer at pH 7.0; 0-40% acetonitrile gradient. Solid line – registration at 245 nm, dashed line - registration at 330 nm.

Fig. 5. Changes in (a) CD spectra and (b) absorption spectra of H_4Bip (0.6×10^{-4} M) in 0.1 M phosphate buffer, pH 7.2 monitored upon UV irradiation. Arrows indicate the changes observed at different wavelengths. Solid line – 0 min, Dashed line - 8min, Dotted line - 16 min, Dash-dotted line - 24 min. Inset show the difference spectrum (24 min – 0 min).

Fig. 6. Optimized geometries of azacyclobutane and cyclobutane H_2Ptr dimers. Method: B3LYP/6-31G(d,p) – COSMO (water). (To see the color in this figure, the reader is referred to the web version of this article.)