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Rate of Tetracycline Photolysis during Irradiation at 365 nm

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Abstract—Kinetics of photolysis of the antibiotic tetracycline (TC) during irradiation at 365 nm was studied in three buffer solutions usually used for studies on TC binding to its main cell targets—a transcriptional repressor protein TetR and to the ribosome. These buffer solutions contain magnesium ions and an antioxidant—mercaptoethanol or dithiothreitol. The rate of TC photolysis was maximal in medium which contained 14 mM mercaptoethanol and 5 mM magnesium ions. In the absence of mercaptoethanol the photolysis rate was more than twofold decreased. The rate constants and quantum yields of the photolysis were determined under various conditions.

Key words: tetracycline, UV absorption spectrum, photolysis, antioxidants, quantum yield, HPLC

Tetracycline (TC) (Fig. 1) is a widely used broad-spectrum antibiotic. However, its use in medicine and veterinary science had become seriously complicated because of the spread of TC-resistant microorganisms. Therefore, production of new TC derivatives is an urgent task of pharmacology [2]. To solve this problem, comparative studies are needed on TC complexes with its targets in the bacterial cell—ribosomes, a tetracycline transcriptional repressor TetR, and the antiporter protein TetA which is responsible for TC transport from the cell. Studies on these complexes are promising for providing important information about the specific interaction of TC with various targets.

X-Ray diffraction is one of the most efficient approaches for studies on structure of complexes, but transmembrane proteins, such as the antiporter protein TetA, are poorly crystallized, and, moreover, this approach cannot be used for studies on protein structure in solutions and for *in vivo* experiments.

The TC spectrum has a clearly pronounced absorption band with a maximum at 365 nm; therefore, a photoactivated affinity modification induced by irradiation at 365 nm can be used to study TC complexes with targets. This approach was used already to modify ribosomes with TC: both rRNA nucleotides [3] and ribosomal proteins [4, 5] immediately adjacent to TC were identified. The antiporter protein TetA was also modified with TC [6]. However, the modification of the proteins in both cases had a very low yield, and the amino acid residues involved in the binding of TC were not determined.

We used photoinduced affinity modification for studies on the structure of TC complex with the TetR(D) protein [7]. In this case the modified peptides were identified, but again it was difficult to analyze in detail and to identify the amino acid residues modified because of the low yield of the modification and the lack of reliable data on the structure of the TC photolysis products.

In particular, the modification could have a low efficiency because of a nonproductive photolysis of TC which competed with the modification of the protein. This is another reason for a careful analysis of the TC photolysis under conditions usual for studies on the modification of its cell targets.

The TC photolysis is additionally interesting because products of TC photodegradation can induce dermatological diseases in patients treated with TC or with its derivatives [8].

In the present work the rate of TC photolysis in different buffer solutions which are usually used for studies on specific complexes of this antibiotic with its targets was

Abbreviations: TC) tetracycline; aTC) anhydrotetracycline; ME) mercaptoethanol; DTT) dithiothreitol; PMR) proton magnetic resonance; HPLC) high performance liquid chromatography; TetR) family of transcriptional repressor proteins. The homology of separate groups is from 40 to 80% [1]. Nomenclature with letters is now widely used, e.g., TetR(D) protein.

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studied as the starting stage for elaboration of the direct photoinduced TC modification of the targets. Three standard buffer solutions were chosen: buffers A and B are usually used in studies on ribosomes, and buffer C is used in studies on the TetR(D) protein. All three buffer solutions contain either ME or DTT, which provide for a mildly reducing medium.

MATERIALS AND METHODS

TC was from Boehringer Ingelheim (Germany), anhydrotetracycline (aTC) was from Janssen Chimica (USA); they were used without additional purification. Other reagents of the maximally available purity were from Serva (Germany). The three buffer solutions used in the work were as follows: A) 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 50 mM KCl, 14 mM ME [4]; B) 20 mM HEPES-KOH (pH 7.6), 3 mM $Mg(CH_3COO)_2$, 150 mM NH_4Cl, 4 mM ME, 2 mM spermine, 0.05 mM spermidine [9]; C) 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 150 mM NaCl, 2 mM DTT [10].

Absorption spectra in the wavelength range of 300-700 nm were measured with a Specord UV-Vis spectrophotometer (Karl Zeiss, Germany). The source of UV radiation was a horizontally placed HQE lamp (OSRAM, Germany) with the main emission band at 365 nm and the electric power of 40 W. TC solutions with the initial absorption A_{370} = 0.9-1.1 were irradiated in horizontally placed quartz cuvettes with covers, with the optical pathlength of 0.5 cm. The lamp was at the distance of 10 cm from the specimens irradiated. The cuvettes were placed onto dark metallic plates about 1.5 cm thick cooled with a mixture of ice with NaCl. Above the cuvettes a glass plate (1 mm thick) was placed which completely absorbed the irradiation with $\lambda < 313$ nm. The specimens were irradiated for 3-6 h, and the absorption spectra were measured every 15-30 min.

The light flux of the HQE lamp was calculated by formula (1) from work [11]:

$$E = \frac{\ln\left(\frac{10^{A_0} - 1}{10^{A_t} - 1}\right)}{\sigma \times \Phi \times t}$$

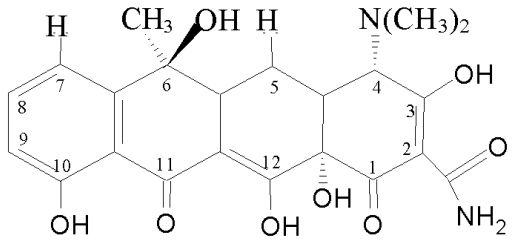
where E is the light power of the lamp (quantum/cm^2 per min); A_o is the initial optical density of the TC solution; A_t is the optical density of the TC solution after irradiation for t min; t is the irradiation time, min; σ is the absorption cross-section (cm^2) of the TC molecule (for TC $\sigma = 9.1 \cdot 10^{-17}$ cm^2); Φ is the quantum yield of TC photolysis.

The quantum yield Φ of TC photolysis in different buffer solutions was calculated by inverted formula (1).

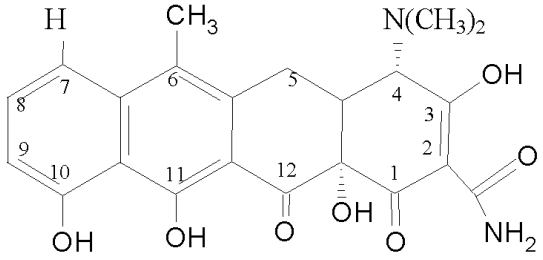
Products of TC photolysis were separated by high performance liquid chromatography (HPLC) on an Ultrasphere C18 column (4.6 × 250 mm) in 0.1% trifluoroacetic acid and acetonitrile gradient from 0 to 30% within 36 min and from 30 to 100% within 30 min. The volume applied was 200 µl. The absorption of the fractions was recorded at 215 and 280 nm.

RESULTS AND DISCUSSION

The UV absorption spectrum of TC has been rather long known to have two main absorption bands with the maximums at 270 and 365 nm [12]. The presence of magnesium ions in the solution results in a red shift of the absorption band with λ_{max} = 365 nm to λ_{max} = 375 nm due to formation of the TC–Mg chelate with involvement of tetracycline oxygen atoms at C11 and C12 (Fig. 1) [13]. Therefore, for the photoaffinity TC modification of targets it is reasonable to use the radiation with the wavelength of about 365 nm. Proteins and nucleic acids do not absorb in this region, thus, their photodestruction and formation of protein–protein and nucleic acid–protein cross-links are prevented. In fact, this approach has been used for TC modification of its targets. But the modification products were significantly different in different



TETRACYCLINE



ANHYDROTETRACYCLINE

Fig. 1. Structural formulas of tetracycline and anhydrotetracycline.

buffer solutions, and sometimes the modification was nonspecific [4]. Thus, it is necessary to study more carefully TC photolysis as it is in different buffer solutions.

Functional complexes of TC with its targets are usually studied in multicomponent buffer solutions. In addition to compounds responsible for the buffer properties these solutions also contain various salts, antioxidants, polyamines, etc. We used three buffer solutions, and their composition is presented in "Materials and Methods".

Buffers A and B are used in studies on ribosomes and buffer C is used in studies on the TetR(D) protein. TC preparations in buffer A, in buffer A deprived of magnesium chloride (A-Mg), in the ME-free buffer A (A-ME), and in buffers B and C were irradiated at the wavelength of 365 nm.

The irradiation increased the absorption of all solutions at 270 nm and decreased it in the region of 370 nm, and new absorption bands also appeared (Fig. 2, the absorption at 270 nm not shown). The irradiation of TC in buffers A and A-Mg resulted in appearance of a rather pronounced new absorption band with the maximum at 439 nm (Fig. 2, a and b), and in buffer A this product was accumulated more rapidly than in buffer A-Mg. According to data of Cooperman et al., this absorption band corresponds to the appearance of aTC, a product of TC photolysis (Fig. 1), and this was confirmed by proton magnetic resonance (PMR) and by chromatography [14]. During irradiation of TC in A-ME buffer the absorption band with the maximum at 439 nm was not detected, but a significantly smaller band appeared with the maximum at 526 nm (Fig. 2c). The irradiation of TC in buffers B and C also resulted in appearance of the absorption band with the maximum at 526 nm (Fig. 2, d and e).

The photolysis of TC during irradiation with "near" UV was studied in some works [4, 15], but the quantum yield of this process was not reported.

Combining data from the works of Cooperman et al. [4, 16] and on assumption that the experiments with photolysis in these works were performed under the same conditions, the quantum yield of TC photolysis in buffer A-ME was calculated with formula (1). The result was $\Phi = 2.5 \cdot 10^{-4}$. Using these data, we determined the light power of our OSRAM HQE lamp to be $2.1 \cdot 10^{17}$ quanta/cm^2 per min, or 18.9 W/m^2. In this case the quantum yield of TC photolysis in our experiments was as follows: $6.5 \cdot 10^{-4}$ in buffer A, $4.1 \cdot 10^{-4}$ in buffer A-Mg, and $2.5 \cdot 10^{-4}$ in the other buffers used. Thus, the photolysis of TC was an order slower than the photolysis of nucleotides, aromatic amino acids, and other biologically important compounds [17-19].

Figure 3 presents changes in the logarithm of the relative optical density of TC solutions ($\ln(A/A_0)$) in the absorption maximum at λ_{375} depending on the irradiation time. This dependence is linear, consequently, the TC photolysis is a first order reaction. The calculated photolysis rate constants are presented in the table.

Obviously, the rate of TC photolysis was maximal in buffer A, the removal of magnesium ions (A-Mg) slightly decelerated the photolysis, and the removal of ME (A-ME) decelerated it still more. The rate constant of TC photolysis in A-ME buffer obtained in the present work is in good agreement with the value of $2.5 \cdot 10^{-3}$ min^{-1} presented in work [4]. The rate constants of TC photolysis in buffers A-ME, B, and C were virtually the same.

Rate constants of photolysis of various TC derivatives were presented in work [15]. In this work, TC photolysis in Hanks' medium (pH 7.0) was induced by irradiation with the intensity of 75 W/m^2. The rate constant of the TC photolysis was found to be $(7.00 \pm 0.02) \cdot 10^{-2}$. Such a significant difference from our data is likely to be due to quite other conditions during the photolysis: another composition of the solution, the higher temperature, the more powerful lamp.

Spectra of TC photolysis (Fig. 2) suggested that the photolysis occurred differently in different solutions as judged by different products generated with new absorption bands. The photolysis rate was maximal when the reaction product was aTC. The comparison of TC photolysis in buffers A, A-Mg, and A-ME suggested that the presence of ME was necessary for production of aTC and that magnesium ions significantly accelerated this production. In A-ME buffer no aTC was formed, in A-Mg buffer aTC was produced at a slow rate, and in A buffer aTC was produced significantly faster than in A-Mg buffer. Because aTC was produced by elimination of the hydroxyl group at the C6 position and of the hydrogen atom at the C5α position, the TC chelate with magnesium ion seemed to stabilize the conformation which was favorable for the elimination, and this, in turn, increased the rate of the reaction.

Products of photolysis in A, A-Mg, and A-ME buffers were preliminarily analyzed by HPLC. The specimens were placed onto the column after 195, 330, and 360 min of the irradiation, respectively. Figure 4 presents a chromatogram of the TC specimen irradiated in buffer A. Non-irradiated solutions of TC and aTC were used as the controls. The fractions *5* and *6* corresponded to the initial TC and the fractions *8* and *9* corresponded to aTC. According to PMR and chromatography data obtained by Cooperman et al., aTC was the main product of TC photolysis in buffer A. Moreover, some minor products of photolysis were accumulated, and one of them was identified as 4-epi-aTC [14].

We compared sets of TC photolysis products in buffers A, A-ME, and A-Mg. In buffer A-Mg fractions *1-4* and fraction *7* were absent, and aTC was the main product of photolysis, in agreement with the spectral data. In buffer A-ME fractions *8* and *9* were absent and no aTC was produced, also in agreement with the spectral data.

The irradiation of TC in buffer A resulted in a whole set of photolysis products which were specific for both the nonreducing (A-ME) and the antioxidative (A-Mg) medium.

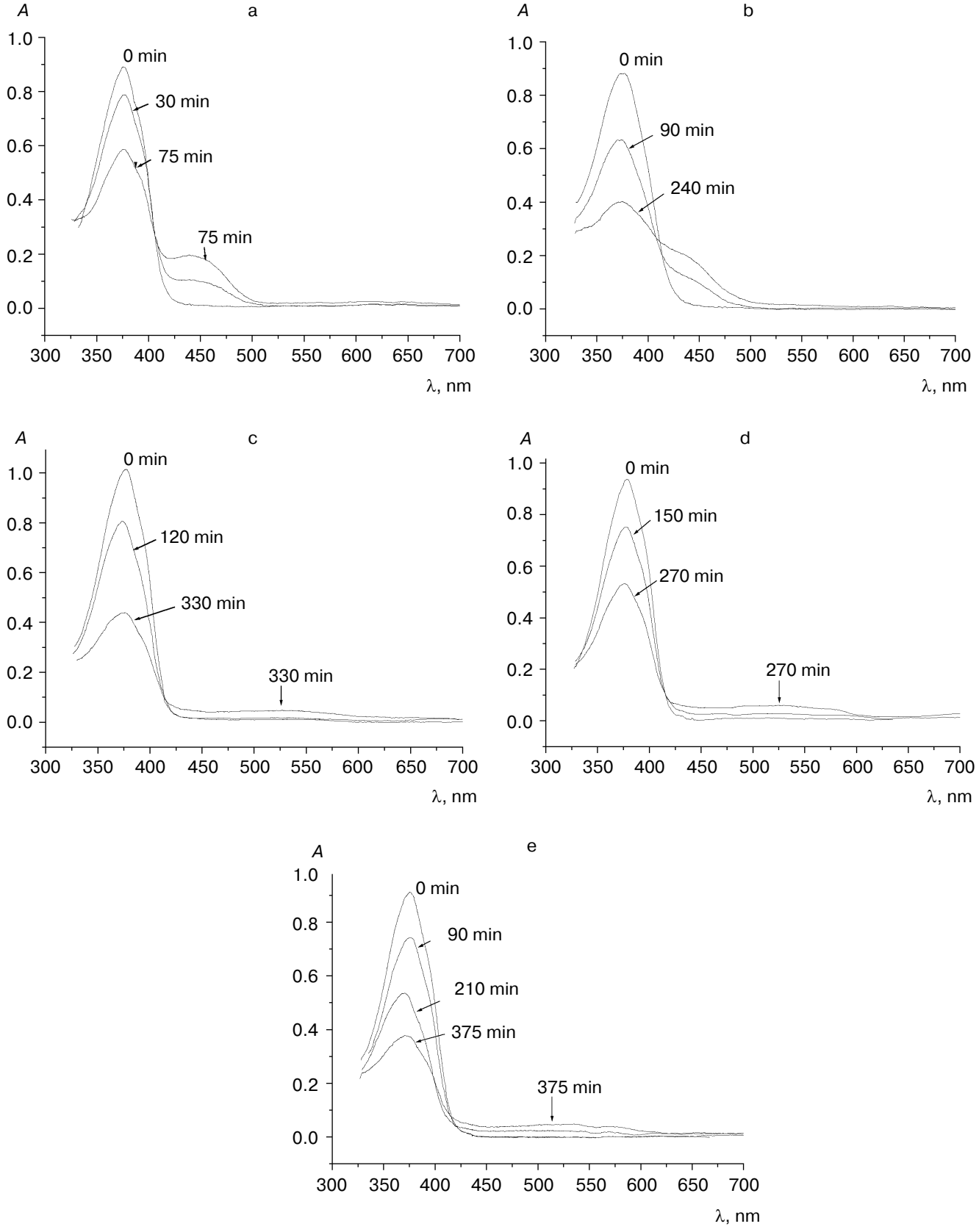


Fig. 2. Absorption spectra of TC in the region of 330-700 nm during irradiation. The values above the curves indicate the time of irradiation: a) in buffer A; b) in buffer A-Mg; c) in buffer A-ME; d) in buffer C; e) in buffer B.

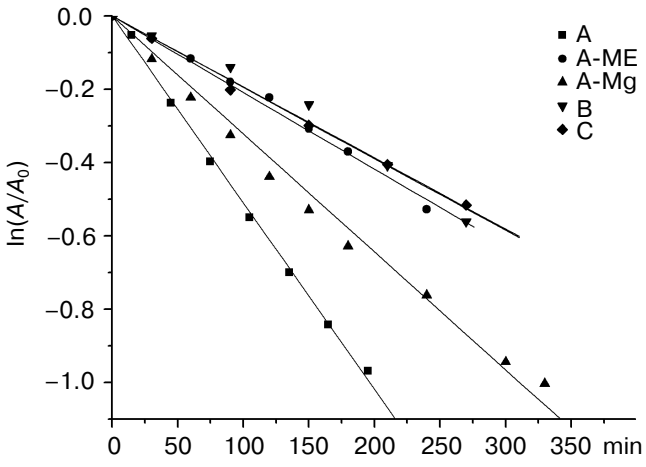


Fig. 3. Time dependence of the $\ln(A/A_0)$ value during TC photolysis in different buffer solutions.

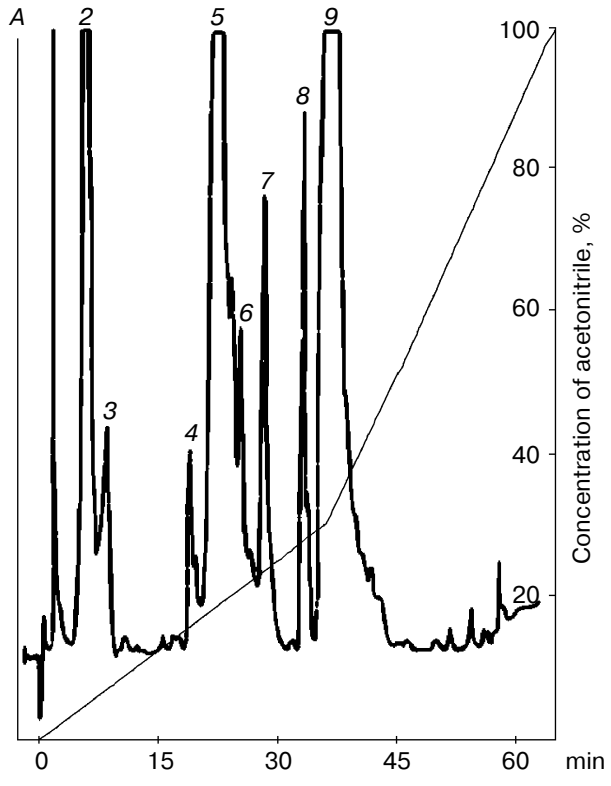


Fig. 4. Separation of TC photolysis products in buffer A by HPLC in an acetonitrile gradient. Fractions *4*, *6*, *7*, and *8* were detected at the higher sensitivity of the detector than it was necessary for the main fractions.

Rate constants of TC photolysis under different conditions

Buffer solution	Rate constant, min^{-1}
A	$(5.10 \pm 0.05) \cdot 10^{-3}$
A-Mg	$(3.22 \pm 0.07) \cdot 10^{-3}$
A-ME	$(2.09 \pm 0.04) \cdot 10^{-3}$
B	$(1.95 \pm 0.03) \cdot 10^{-3}$
C	$(1.94 \pm 0.08) \cdot 10^{-3}$

This suggested that the presence of magnesium ions in buffer A, on one hand, increased the rate of aTC accumulation and, on the other hand, stimulated another pathway of photolysis associated with the generation of products specific for the non-reducing medium. Their minor quantity as compared to that of aTC explained the absence of a pronounced absorption band at 526 nm in buffer A, although a slightly increased absorption in this region was recorded (Fig. 2a). The presence of ME determined the main pathway of the reaction resulting in aTC production.

During photolysis in buffer B a new product was generated with absorption at 526 nm, but the TC photolysis rate under these conditions was rather low. However, buffer B, similarly to buffer A, contained ME and magnesium ions. This phenomenon seemed to be explained as follows.

The TC photolysis in oxygen-containing solutions is known to produce singlet oxygen [20] which is involved in the generation of photolysis products. The presence of ME or other SH compounds prevented accumulation of singlet oxygen [21]. Buffer B contained only 4 mM ME, and this seemed to be insufficient for binding all the singlet oxygen produced because the oxygen concentration dissolved at 4°C is ~2 mM.

Moreover, buffer A contained a base (Tris) as a buffer compound, whereas the buffer compound in buffer B was an acid (HEPES). We also performed TC photolysis in a buffer which contained only 50 or 110 mM Tris-HCl (pH 7.6). On changing to the buffer with a high content of Tris-HCl the rate constant of TC photolysis increased from $2.5 \cdot 10^{-3}$ to $1.1 \cdot 10^{-2}$ min^{-1}, i.e., more than forty times, that indicated an important role of the buffer compound in the photolysis. And the TC photolysis rate in 50 mM Tris-HCl buffer (pH 7.6) was comparable to the rate in buffer A-ME, and in 110 mM Tris-HCl buffer (pH 7.6) its rate was higher than in buffer A, although the product with absorption at 526 nm was produced (data not presented).

Buffer C contained 2 mM DTT instead of ME and, unlike the other buffers, had a higher pH value of 8.0. The rate constant of TC photolysis in this buffer was the same as in buffer B. During photolysis a product was generated with absorption maximum at 526 nm. The concentration

of the antioxidant DTT in buffer C was 2 mM, and this could be insufficient to neutralize the singlet oxygen generated during the photolysis. The structure of the product generated during TC photolysis under antioxidative conditions is still not defined unambiguously: deamination of the TC dimethylamino group has been supposed [22, 23].

Thus, aTC was the main product of TC photolysis in buffers with a high content of ME. The presence of magnesium ions in the solution results in two effects: an increase in aTC accumulation rate and the generation of products specific for oxidative conditions. Both aTC and photolysis products generated under oxidative conditions are likely to produce artifacts during photoaffinity modification, e.g., nonspecifically modify ribosomal proteins [4, 5]. In other words, if the photolysis rate was too rapid, the probability of a nonspecific modification of TC increased. Consequently, to study the UV-induced TC modification of its targets the conditions are to be chosen with respect to specific features of TC photolysis.

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