Thus, the error in determining the position of the intensity maximum is 0.1%, the error in determining the width is 3.7%, and the error in determining the form is 3.2%; this corresponds to the results obtained in testing the model spectrum.

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CHROMATOGRAPHIC AND SPECTROSCOPIC INVESTIGATION OF THE PRODUCTS OF OXIDATION OF TYROSINE WITH OZONE

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The establishment of the molecular mechanisms of the toxicity of ozone is one of the fundamental tasks in the study of the action of ozone on biological materials.

In previous work [1] it has been shown that ozone in low concentrations readily oxidizes aromatic amino acids both in solution and when constituents of proteins resulting in the formation of a whole series of oxidation products. Since the oxidation products are physiologically active it was of interest to study them further. In the present work the main molecular products obtained by oxidation of tyrosine with ozone are established, and possible mechanisms of their formation discussed.

The materials used in the work were tyrosine supplied by Reanal (Hungary), 3,4-dihydroxyphenylalanine (DOPA) from Sigma (USA), 3,3-dityrosine prepared by the method in [2]. Tyrosine solutions were prepared of 0.1% concentration in 0.02 mole/liter phosphate buffer pH 7.4. Ozone was prepared and purified as described previously [1]. The concentration of ozone in the gas phase was monitored spectrophotometrically and maintained at 2.5×10^{-5} mole/liter. Treatment of the tyrosine solutions with ozone was effected by bubbling a stream of the ozone–air mixture through a 10-ml volume at a rate of 0.2 liter/min. Gel-filtration of the ozonized tyrosine solutions through a 1.55 × 55 cm column of Sephadex G-10 (supplied by Pharmacia, Sweden), in equilibrium with the original buffer, was carried out. Three milliliters of the ozonized tyrosine solution was introduced into the column and the oxidation products eluted with buffer at a flow rate of 30 ml/h. Desorbed fractions, each of 3 ml, were analyzed spectrophotometrically at wavelengths of 290 and 240 nm, and spectrofluorometrically at $\lambda_{excit.}$ = 296 and 320 nm. In order to obtain purer preparations of the products the dimensions of the chromatographic column were increased to 1.5 × 87 cm. This enabled the desired separation to be achieved and their spectroscopic identification to be made. The absorption spectra of the preparations were recorded on a SF-16 spectrophotometer, and the fluorescence spectra were recorded on the instrument described previously [1]. The IR spectra of the samples in KBr tablets were recorded on a IR-75 spectrophotometer (GDR).

The ozonized tyrosine solutions were subjected to gel-filtration on Sephadex with the object of separating its oxidation products with different molecular weights. In Fig. 1 the results are shown of the chromatography of ozonized tyrosine. Spectrophotometric recording of the eluates (wavelength 290 nm) permitted the detection of product I, emerging in the free volume of the column which indicates its high molecular weight, as well as fraction II, which corresponds predominantly to nonozonized tyrosine. The appearance should be noted of the low

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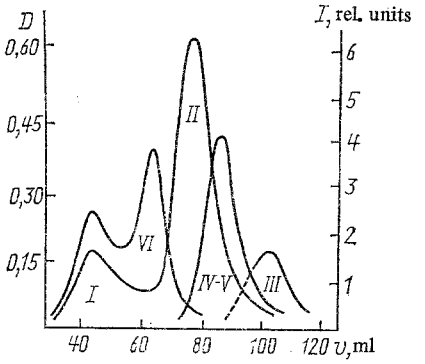

Fig. 1. Composition of the products of oxidation of tyrosine by ozone when resolved on Sephadex G-10. Appearance of products: I, II, III — spectrophotometrically; IV, V, VI — spectrofluorometrically (λ = 290 (I, II) and 240 nm (III); $\lambda_{excit.}$ = 296 (IV) and 320 nm (V, VI)). Column 1.5 × 55 cm.

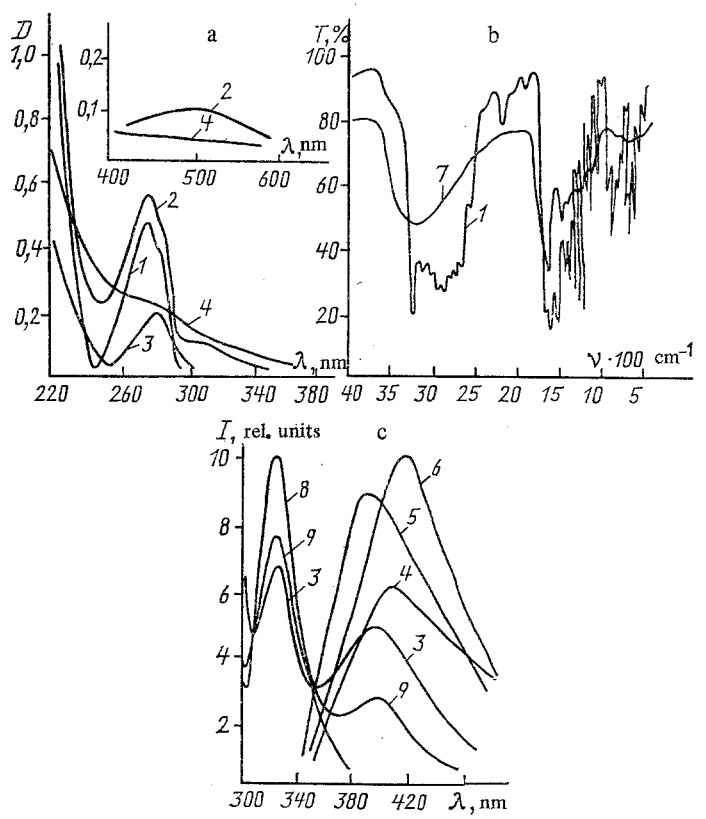

Fig. 2. (a) Absorption, (b) IR, and (c) fluorescence spectra of the products of oxidation of tyrosine with ozone after separation on a 1.5 × 87 cm Sephadex G-10 column. Designation of products as in Fig. 1. 1) Tyrosine (product II), 2) tyrosine after treatment with 5×10^{-5} mole ozone, 3) product IV, 4) product I, 5) product V, 6) product VI, 7) product VII, 8) 3,4-DOPA ($C = 1 \times 10^{-4}$ mole/liter, 9) 3,4-DOPA after treatment with 1×10^{-5} mole ozone. $\lambda_{excit.}$ = 296 (3) and 320 nm (4-6).

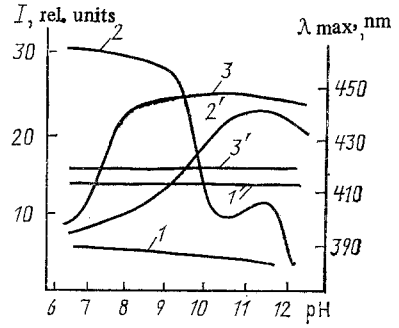

Fig. 3. Spectrofluorometric titration of the products (1) I, (2) V, (3) VI in the alkaline pH region ($\lambda_{excit.}$ = 320 nm). 1-3) change in intensity, 1'-3') shift of λ_{max} of the fluorescence spectra of the products during titration.

molecular weight fraction III, which is eluted after tyrosine and absorbs light in the region below 240 nm. The absence of an aromatic ring in the structure of the molecules of this fraction, as well as the shift of the pH values of the eluates towards the acid region, can indicate the formation of organic acids during ozonization of tyrosine. By using spectrofluorometric recording of the eluates with excitation of the fluorescence of the solutions beyond the longwave absorption band of tyrosine, it was possible to additionally detect the products IV, V, VI (Fig. 1). During separation of the tyrosine oxidation products a compact fraction VII appeared which was black in color and stayed set in the upper part of the column due to the relatively large size of the particles which formed.

Thus gel-filtration with simultaneous application of spectrophoto- and spectrofluorometric recording of the eluates resulted in the recording of seven ozone oxidation products of tyrosine, possessing both higher and lower molecular weights compared with that of tyrosine.

The products of the ozonization of tyrosine isolated by gel-filtration were identified subsequently by spectroscopic methods and application of chemical tests characteristic for the assumed compounds [3].

The presence of indole chromophores in the molecular structure of fraction VII was demonstrated by carrying out the color reaction for indole compounds. However the absorption spectrum of product VII in 0.5 N alkali solution possesses a weak band in the region of 280 nm (Fig. 2a), which can indicate conjugation of the chromophores in the structure of the product. Comparison of the IR spectra of nonozonized tyrosine and product VII (Fig. 2b) showed that in the spectrum of the product the number of vibrational bands had decreased and the bands themselves were broadened. Vibrational spectra of this character are typical of polymers [3]. The characteristic vibrational bands of product VII are: a strong, broad band with maximum 3200 cm^{-1}, due to stretching vibrations of the NH (OH) groups participating in hydrogen bond formation; a band with a maximum at 1640 cm^{-1}, which is probably due to the vibrations of aromatic ring double bonds, as well as bands at 1380, 1230, 1100 cm^{-1}. The vibrational spectrum obtained for product VII corresponds to the known IR spectra for melanin [4]. The enumerated spectral and chemical properties, as well as the presence of an ESR signal due to unpaired electrons with $g \cong 2$ which was recorded on our instrument permits product VII to be considered to be melanin.

Product I had the same absorption spectrum as product VII but in distinction from it was colored yellow and remained in aqueous solution. This can indicate a relatively lower molecular weight of product I compared with product VII. A distinguishing property of product I also is its ability to fluoresce with a spectrum maximum at 411 nm (Fig. 2c), there being no ionizable hydroxyl groups present in the composition of product I, as shown by spectrofluorometric titration (Fig. 3). It can be assumed that this compound is formed as a result of incomplete condensation of tyrosine oxidation products to melanin.

Product IV was characterized by a UV absorption with a maximum at 280 nm and fluorescence with a maximum at 325 nm (Fig. 2a, c). Analogous spectral properties are characteristic for DOPA. The formation of this compound has been chromatographically observed in the oxidation of tyrosine with ozone in another study [5]. As known DOPA is the primary product in the multistage oxidative conversion of tyrosine to melanin. Its subsequent oxidation results in the appearance of dopaquinone, dopachrome and indole-5,6-quinone [6]. The latter can be visually observed from the coloration of the tyrosine solution by a reddish purple color after ozonization, as well as the appearance in its absorption spectrum of new bands in the UV (290-340 nm) and the visible (420-580 nm) regions of the spectrum (Fig. 2a).

Emerging jointly with DOPA from gel filtration is product V, the maximum of the fluorescence spectrum of which is found at 394 nm (Fig. 2c). Fluorescescent titration of product V solution showed that its fluorescence is quenched in the alkaline pH region, with which a shift of the maximum of the fluorescence spectrum from 394 to 440 nm is observed (Fig. 3). With increase of the pH of the solution or on heating the product the solution becomes colored yellow and the molecular weight is increased which was established by repeat chromatography. The instability in solution, the ability to condense as well as a comparison of the obtained spectral characteristics with the behavior of DOPA during its oxidation with ozone (Fig. 2c) permits product V to be identified as dopaquinone.

Product VI, the fluorescence spectrum maximum of which is situated at 415 nm (Fig. 2c), has a higher molecular weight than tyrosine (Fig. 1). Use of spectrofluorometric titration permitted the presence of ionizable hydroxyl groups in the structure of product VI to be established and a structure of the compound formed to be proposed (Fig. 3). It is known that with ionization of tyrosine its fluorescence is quenched, on which a shift is observed of the fluorescence spectrum maximum to 348 nm [7]. Products of the oxidation of tyrosine, dityrosine and trityrosine, behave differently. The intensity of their fluorescence sharply increases with pH of the medium, corresponding to their ionization regions, during which no shift of the maximum of the fluorescence spectra is observed. For dityrosine pK_1 of the transition is at 7.5, pK_2 lies above 13.0 and for trityrosine these values are 6.0 and 12.0 respectively [8]. As follows from Fig. 3, the intensity of the luminescence of product VI is increased in the range pH 7-8, further change as well as shift of the fluorescence spectrum maximum not occupying up to pH 12.1. The spectral properties of product VI do not change when its solution is held at 100°C for 30 min. All this can indicate that in the oxidation of tyrosine with ozone a dimer of the C—C type is formed, 3,3-dityrosine.

By using known views of the mechanism of oxidation of phenolic compounds by ozone [9], and taking into account the experimental results, the following scheme can be put forward for the formation of the molecular products I-VII during oxidation of tyrosine. Ozone is able to attack tyrosine at several sites. With addition of ozone to the phenol ring of tyrosine it is possible for its aromatic ring to cleave and the formation of low molecular weight products, acids and aldehydes, to occur. The given process apparently leads to the formation of fraction III. Ozone is also able to induce separation of the hydrogen atom from the hydroxyl group of the phenol ring in the molecule, which leads to the appearance of phenoxyl and hydroxyl radicals [9]. One path by which phenoxyl radicals disappear is their reaction with one another and the molecules of the original phenolic compound, resulting in the formation of polymeric phenolic compounds of the C—O and C—C type and in particular dityrosine. The hydroxyl radicals, which are formed either during decomposition of the intermediate complex of ozone with the phenol compound, or as a result of the reaction of ozone with water, can add to the phenol ring of the molecules. This process apparently leads to the appearance of DOPA, the subsequent oxidation of which gives rise to the formation of dopaquinone, dopachrome and finally to melanin. During oxidation of tyrosine with ozone decarboxylation and deamination in the tyrosine side chain can occur, however the results of these processes were not recorded by the methods that were used.

Thus the study which has been carried out of the molecular products of the ozonization of tyrosine indicates the formation of products of a quinonoid and biphenolic nature, as well as their condensation products. This result can serve as a basis for the explanation of the molecular mechanisms of crosslinking of proteins under the action of ozone.

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SPECTROSCOPIC STUDY OF TRANSFORMATION OF OXYHEMOGLOBIN AT 293 AND 77°K UNDER THE EFFECT OF UV LIGHT

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It is known that methemoglobin is the main photochemical product formed after UV irradiation in the 260-300-nm region of aqueous solutions of oxyhemoglobin, the main component of blood erythrocytes [1-3].

It was found that under the action of the UV light in doses of $0.45\text{-}4.53\cdot10^6$ erg/cm^2, the heme–protein bonds in oxyhemoglobin of mice become weakened [4].

No photooxidation of the hemoproteid molecules with transition of the iron atom into the trivalent state is observed when hemoproteid solutions are irradiated by long-wave UV light in the range of 320-390 nm [3]. The value of the activation energy of the photochemical oxidation of oxyhemoglobin varies with variation in the doses of UV radiation: Increase in dose leads to a marked decrease in the activation energy of this photoprocess [4, 5].

However, the quantitative characteristics of the occurrence of the photochemical reaction of the formation of methemoglobin have been little studied, in particular, with respect to finding general regularities of the formation and accumulation of free radicals during UV irradiation of the oxygenated form of hemoglobin.

To reveal these regularities, we must understand the mechanism of the action of UV radiation on erythrocytes and blood itself, recently widely and successfully used in clinical medicine for treating various diseases.

In the present work we report the results of the kinetic investigation of the formation of methemoglobin in oxyhemoglobin solutions irradiated by UV light under different temperature conditions: The degree and quantum yield of the photooxidation of oxyhemoglobin molecules at 293°K and also the values of the quantum yields of formation of free radicals in aerated and evacuated solutions (77°K) of this two-component protein were determined.

Oxyhemoglobin was isolated from the blood of white mice by the method described in [6].

The degree of oxidation of oxyhemoglobin molecules [2] after UV irradiation of its aqueous solutions through a UFS-1 light filter ($2\cdot10^{-6}$ mole/liter, pH 5.80, temperature 20°C) was calculated from the formula

$$Mt\,\text{Hb},\ \% = \frac{100\cdot(D_x - D_1)}{D_2 - D_1},$$

where D_X is optical density at 630 nm of irradiated solution of oxyhemoglobin; D_1 is optical density of initial solution of hemoproteid at 630 nm; D_2 is optical density of oxyhemoglobin solution after addition of one drop of a 20% solution of potassium ferricyanide to 5 ml of protein.

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