Effect of the Concentration of Humic Acids in Aqueous Solutions on the Structure of Their Macromolecules

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Abstract

The concentration dependence of the first moments M_1 of fluorescence spectra of the fractions of humic acids (HA) differing in molecular masses in aqueous solutions within the concentration range 0.5–50 mg/l is investigated correctly for the first time. For all the HA fractions investigated, up to the concentration of ~5 mg/l, a substantial increase in M_1 value is discovered. It is established that M_1 value undergoes smaller changes with an increase in the molecular mass of HA fraction. Interpretation of the concentration dependence of M_1 is carried out with the help of the hypothesis of associates. Within this hypothesis, the observed batochromic shifts of the fluorescence spectra of HA fractions with an increase in concentration are explained by the changes in the conformation of the fluorescing substance and by changes in the polarity of microsurroundings.

INTRODUCTION

The urgency of the investigation of macrostructure of humic acids HA in aqueous solutions is due to the fact that only humic substances possess the entire set of properties that create specific ecological conditions of global importance in the water reservoirs of the Earth. In addition, investigation of the structural status of HA macromolecules in an aqueous medium is timely since it is HA that promote degradation of dissolved pollutants (amines, phenols, heterocyclic compounds, heavy metals, *etc.*) due to binding them in natural water to form various complex compounds [1].

The structure of HA macromolecules has been studied for about 200 years but it is still the subject of discussions. Till middle 90-es of the 20 century, the most widespread opinion (generally accepted paradigm) on the structure of HA molecule was as follows: there is a nucleus (aromatic carbon framework) and a periphery (polysaccharide-polypeptide chains) [1]. The molecular fragments of the nucleus and the periphery of the same HA molecule are bond with each other by chemical bonds. Bearers of the specific characteristics of humic acids are the condensed aromatic nuclei connected with each other by covalent bonds. Peripheral irregular structural elements (peripheral chains) are variable components; therefore, a HA is a charged polymer (polyanion) with the molecular mass up to 300 000 kD which can be varied. So, HA macromolecules are characterized by a wide set of various structures, that is, they are polydisperse.

Until [2], different hypotheses concerning the macrostructure of HA in aqueous solutions were available in literature. For instance, the authors of [3–5] believe that HA are spherical colloids, or globular formations, while the authors of [6–8] assume that the macrostructure of HA is a flexible chain polymer having polyelectrolyte nature. There were attempts to conciliate the opposite hypotheses assuming that the macrostructure of HA is a mixture of spherical colloids and linear polymers [9, 10]. However, this hypothesis did not obtain recognition. The authors of [2] tried to propose a unified concept that could clarify understanding of the struc-

ture of HA. For this purpose, they carried out a systematic investigation of the concentration dependences of viscosity coefficient and surface tension of the aqueous HA solutions at different ionic force and pH. As a result, the authors of [2] concluded that the macrostructure of HA in low concentrations in alkaline media is a flexible linear polymer, while at high concentrations it is a rigid spherical colloid. However, the mechanism of the formation of a rigid spherical colloid (for example, due to association or twisting of the initial monomer molecule) was not described in [2].

In connection with the problem of HA conformation in aqueous solutions, amphiphilic properties of these molecules should be stressed. This is explained by the presence of polar and non-polar fragments in the structure. Due to this fact, the formation of supramolecular complexes becomes possible due to self-association. The occurrence of these processes was established, for example, for biological membranes [11] a simpler molecules [12]. It is amphiphilic character of HA that is the basis of an alternative hypothesis about the structure of HA molecules in aqueous solutions, proposed in 1996 [13]. Its essence is that a HA macromolecule is not a polymer with covalent bonds between all the atoms in a molecule but a supramolecular micelle-like aggregate which is composed of relatively small heterogeneous molecules with the molecular mass up to 1000 D; the aggregates are able to undergo self-association due to weak dispersion forces (Van der Waals interactions, CH–p-, p–p complexes) and H-bonds [13, 14]. It should be stressed here that [13] was preceded by [15] in which it was pointed for the first time to weak hydrophobic interactions as the factor promoting the formation of the structure of HA molecule in solution. So, a principal provision of the supramolecular model is the absence of covalent bonds between relatively small molecules.

Fluorescence method has recently become one of the simple and generally accepted methods of the investigation of structural characteristics of HA [16–19]. This method allows one to obtain extensive information about changes in the structure of fluorescing groups in molecules depending either on the changes in the own conformations of HA molecules [16, 18, 21, 29] or on polarity of their microsurroundings [16–20]. The fluorescence spectra of HA are non-uniform because they are a superposition of different fluorescing groups belonging, due to polydispersity, to different HA molecules [17, 22]. The quantitative parameters obtained in the observations of fluorescence spectra are integral intensities, first moments M1 ("centre of gravity" of a spectrum) [16, 17], coefficients of the shape of fluorescence spectrum (a ratio of the intensity of the shorter- and longer-wavelength components of the fluorescence spectrum) [17, 21, 22, 24].

Systematic investigations of the macrostructural properties of HA in aqueous solutions depending on concentration have not been carried out by means of fluorescence method, only a few works are known (see, for example, [23, 30, 31]). Among the results directly related to the problem of investigation of HA macrostructure depending on concentration, only the data of [30] can be mentioned; the authors observed a batochromic shift of HA fluorescence spectrum with an increase in HA concentration. The authors expected that the shift was connected with the possible formation of an aggregated state of HA macromolecules.

However, many works (not only those indicated above) in which HA fluorescence spectra were investigated contain some errors making it difficult to obtain the reliable information about HA macrostructure. These errors are due to the absence of corrections to HA fluorescence spectra for the inner filter effect (IFE) [32, 33]. The essence of this effect is that the optical density at the wavelengths of fluorescence observation is different from zero and depends on concentration. Another error is connected with the absence of the account of the fluorescence of the solvent, which is always observed in aqueous solutions [21]. So, at present, there is no reliable information on changes in the fluorescence spectra of HA molecules (and therefore on changes in their structure or surroundings) depending on concentration. Meanwhile, the urgency and importance of obtaining such information are brought about by the additional interest in view of determination of the constants of HA binding with various pollutant molecules. Such a determination is carried out

on the basis of analysis of experiments aiming at establishing the dependence of fluorescence intensity from pollutant molecules on HA concentration (determination of Stern–Volmer constants) [34–36]. In doing this, it is accepted that the structural properties of HA are independent of concentration, though this is not evident *a priori*.

The data on the effect of the mass of HA fraction on the concentration dependencies of fluorescence spectra are completely absent, though the results of these experiments would be very important for solving the problems connected with the investigation of both the interaction of HA molecules in aqueous solutions and the molecular structure and functional characteristics of HA itself.

The goal of the present work was to investigate the effect of concentration on the macrostructure of the fractions of HA extracted from the same genesis, with the help of fluorescence method. As it was mentioned above, this method is a sensitive tool to monitor changes in the structural parameters of HA [16–31, 34–36]. We chose the first moment as the spectral parameter extracted from the observed changes in fluorescence spectra. The possibility to use this parameter to obtain the data on structural changes in HA macromolecules was successfully demonstrated previously [18, 19].

EXPERIMENTAL

The samples were HA fractions extracted from the soil of one kind (black soil from the Kursk Region). Extraction of HA and preparation of the fractions designated as C + D, B + C, B and A were described in detail previously [37–39]. Molecular masses determined with the help of ultrafiltration method were 5–30, 30–60, 60–100 and 100–300 kD, respectively [38]. The alkaline solutions were prepared from dry HA using deionized water (R = 6 MW); the acidity of solutions was pH 12.3 (NaOH "ch.d.a." reagent grade). The solution pH did not change with variations of HA concentration.

Fluorescence spectra were recorded in 24 h after the solution was prepared. No changes were observed in absorption and fluorescence spectra during the storage of solutions from one record to another (3 days as a maximum). Fluorescence spectra were recorded with a N_2 laser strobofluorimeter (λ_{excit} = 337 nm) [40]. Laser beam passed through the quartz cell (1 × 0.5 × 1 cm) from above, parallel to the entrance slit of the fluorimeter. The centre of the excitation laser beam 3 mm in diameter was situated at a distance of 2 mm from the front edge of the cell. The cell was filled with the solution so that no meniscus was seen. Experiments were carried out with the samples from which air was not removed. The absorption spectra of HA solutions were recorded with a Hewlett Packard spectrometer.

The first moment M_1 of fluorescence spectra of HA fractions was determined as

$$M_1 = \Sigma I_i \lambda_i / \Sigma I_i \quad (1)$$

where I_i is the intensity of luminescence at the wavelength of λ_i, which was calculated suing equation

$$I_i = I_{i\,\mathrm{HA}}^{\mathrm{exp}} \cdot 10^{\mathrm{OD}_{\lambda_i}} - I_{i\,\mathrm{alk}}^{\mathrm{exp}} \quad (2)$$

Here $I_{i\,\mathrm{HA}}^{\mathrm{exp}}$, $I_{i\,\mathrm{alk}}^{\mathrm{exp}}$ are experimentally observed intensities of fluorescence at the wavelength of λ_i of HA and alkali solution, respectively; $10^{\mathrm{OD}_{\lambda_i}}$ factor is a correction for fluorescence absorption in HA solution at the wavelength of λ_i; OD_{λ_i} is the optical density of the sample under investigation at the wavelength of λ_i for the optical length of 2 mm. The correction taking into account absorption at the excitation wavelength which is 337 nm (factor $(1 - 10^{-\mathrm{OD}_{337}})^{-1}$) was not introduced to both terms of the right-hand part of equation (2) because taking it into account, as follows from (1), has no effect on M_1 value. Decomposition of the contours of HA fluorescence spectra into components was carried out with standard software (Origin 6).

RESULTS AND DISCUSSION

Absorption spectra

The absorption spectra of B + C fraction depending on HA concentration are shown in Fig. 1. Similar dependencies were also observed for other fractions. One can see in these data

that concentration changes have no effect on the shape of absorption spectra. Insert in Fig. 1 shows the concentration dependencies of OD values at several wavelengths for B + C fraction. One can see that Bouguer–Lambert–Beer law (BLB) clearly holds true with these data. For other fractions at different wavelengths, also linear dependencies of OD on concentration were observed. The obtained dependencies agree with literature data [1]. Realization of the BLB law within the studied concentration range means that the properties of the assembly of chromophores that form absorption spectra of HA macromolecules are not changed with changes in solution concentration.

Fluorescence spectra

Effect of inner filter effects and dissolved oxygen on fluorescence spectra. It was shown previously [41] that in order to take into account the IFE in emission spectra accurately, it is necessary to take into account (OD ~ 0.8) the finite geometric width of the excitation laser beam. This is due to the fact that the $10^{OD_{\lambda_i}}$ correction (most recommended correction for the IFE involving BLB law) can be used correctly only in the case of infinitely narrow exciting beam. in our experiments within the HA concentration range 0.5–50 mg/l, the OD value did not exceed 0.4, so we could neglect the difference in corrections for IFE calculated with the BLB law and those obtained with accurate calculation. Indeed, for example, M_1 values for C_{HA} = 55 mg/l of B fraction calculated with the help of correction according to BLB and accurate calculations were 466.5 and 466.6 nm, respectively. This difference is approximately an order of magnitude smaller than the accidental experimental error of M_1 determination. It should also be noted that within the HA concentration range 0.5–5 mg/l in which M_1 changes are most substantial (see Fig. 4 below) corrections for absorption can be neglected because for these concentrations the introduction of a correction of the IFE does not exceed 3 % for the absolute integral intensity and 0.2 nm for M_1.

Results of the analysis of experiments on comparison of HA fluorescence spectra with argon bubbling and without it showed that the parameters of HA fluorescence spectra (integral intensity, contour shape) coincide. This could be expected because typical HA fluorescence time is not more than several nanoseconds [23, 42], so the presence of O_2 (with its concentration in water ~2.9 10^{-4} M [43]) cannot

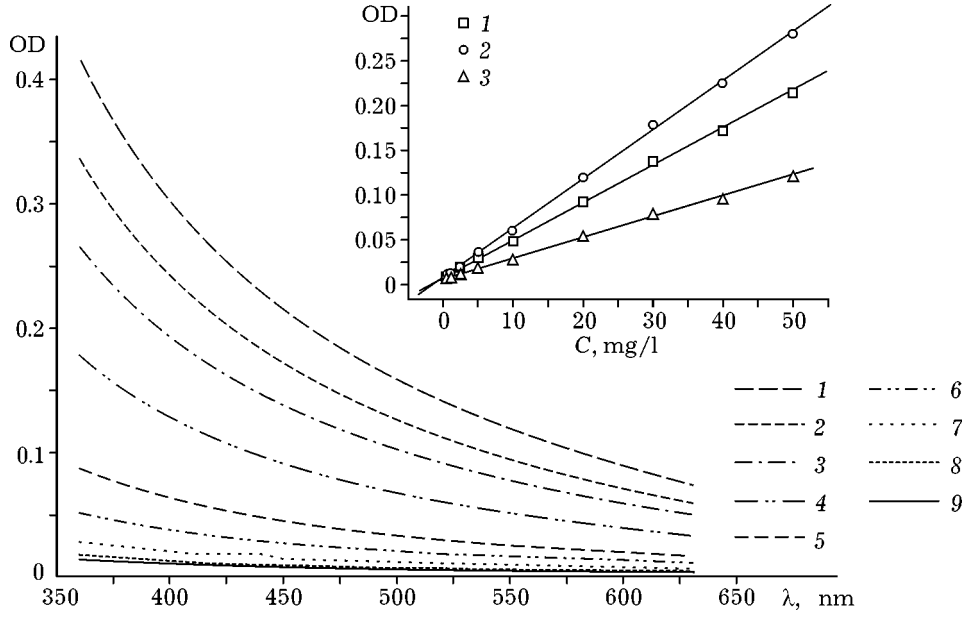

Fig. 1. Concentration dependencies of absorption spectra for a sample of fraction B + C. d = 0.5 cm; T = 295 K; HA concentration, mg/l: 0.5 (*1*), 1 (*2*), 2.5 (*3*), 5 (*4*), 10 (*5*), 20 (*6*), 30 (*7*), 40 (*8*), 50 (*9*). Insert: Dependence of the optical density of the sample of fraction B + C on concentration; wavelength, nm: 410 (*1*), 450 (*2*), 550 (*3*).

cause any noticeable effect on the spectral parameters of HA fluorescence in aqueous solutions.

Dependence of the fluorescence spectra of HA fractions on concentration. Experimentally observed fluorescence spectra of C + D fraction for several concentrations are shown in Fig. 2, *a*; fluorescence spectra for the same concentrations, corrected according to equation (2), are shown in Fig. 2, *b*; normalized fluorescence spectra shown in Fig. 2, *b* are presented in Fig. 2, *c*. One can see that a clear growth of a shoulder at $\lambda \sim 490$ nm is observed fort his fraction with an increase in HA concentration. Similar transformations of the spectrum – an increase in the contribution from the red-side part of spectrum with an increase in HA concentration – were observed also for other fractions.

Decomposition of the contours shown in Fig. 2, *b* into two components (Fig. 3) shows that an increase in concentration causes an increase in the longer-wavelength component. In addition, an increase in concentration causes a long-wavelength shift of all the components (see insert in Fig. 3). An increase in the intensity of the long-wavelength component and the long-wavelength shift of both components with an increase in HA concentration should cause an increase in M_1 of the total fluorescence spectrum, which is indeed observed experimentally (Fig. 4). The observed increase in M_1 may be due to two reasons: an increase in the contribution from the wavelength component into total intensity, and the long-wavelength shift of all the components. It should be noted that an increase in M_1 may be due only to an increase in the contribution from the long-wavelength component, even if the positions of maxima of fluorescence spectra of all the components are independent of concentration.

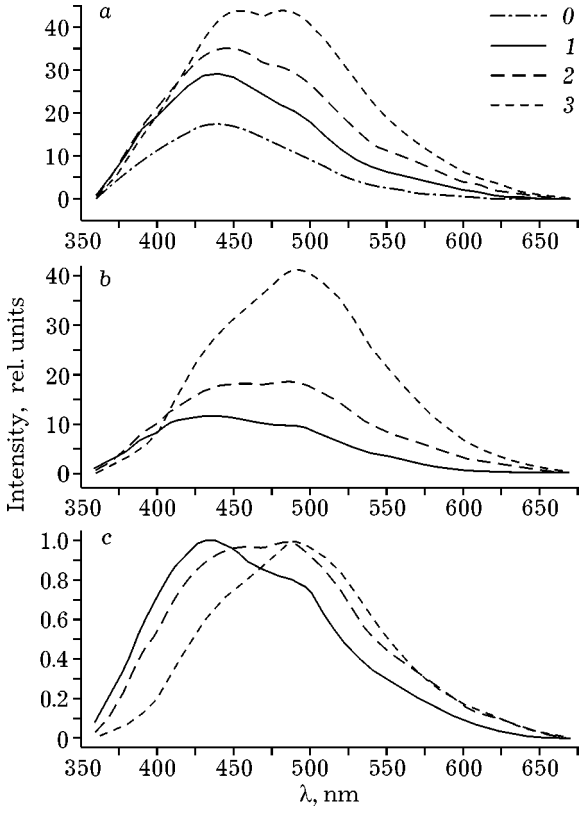

Fig. 2. Fluorescence spectra of the fraction C + D (T = 295 K): *a* – experimental; *b* – corrected using equation (2); *c* – normalized corrected spectra; *0* – fluorescence spectrum of alkaline solution; C_{HA}, mg/l: 0.5 (*1*), 1 (*2*), 2.5 (*3*).

One can see in the data shown in Fig. 4 that the concentration dependencies of M_1 can be of two kinds: with a plateau (fractions C + D, B, A) and without a plateau (fractions B + C, ungraded HA). In addition, non-uniform increase in M_1 with an increase in HA concentration is characteristic of all the fractions: the most substantial changes in the first moment is observed for concentrations up to ~5 mg/l.

An increase in M_1 with an increase in concentration was also observed for aqueous solutions of HA at pH 6.5 (C_{NaOH} = 0, ungraded sample). This fact provides evidence that the observed shifts are not a consequence of the interaction of HA with impurities that can be present in an alkali. So, let us stress once more that it is an increase in HA concentration that causes the long-wavelength (batochromic) shift of the fluorescence spectrum.

The absolute values of M_1 for $C \sim 50$ mg/l (see Fig. 4) differ for different fractions. This may be explained if we accept that the fluorescence spectra of different HA fractions are due to the fluorescing groups of different chemical nature. Indeed, it was shown in [37] that an increase in the fraction mass is accompanied by an increase in the fraction of high-molecular homologues of fatty acids; it was established in [38] that the concentration of amino acids in the lowest in the fraction C + D. Finally, the authors of [[44] analysing the data obtained from the electron and IR absorption spectra and ^{13}C NMR conclude that HA frac-

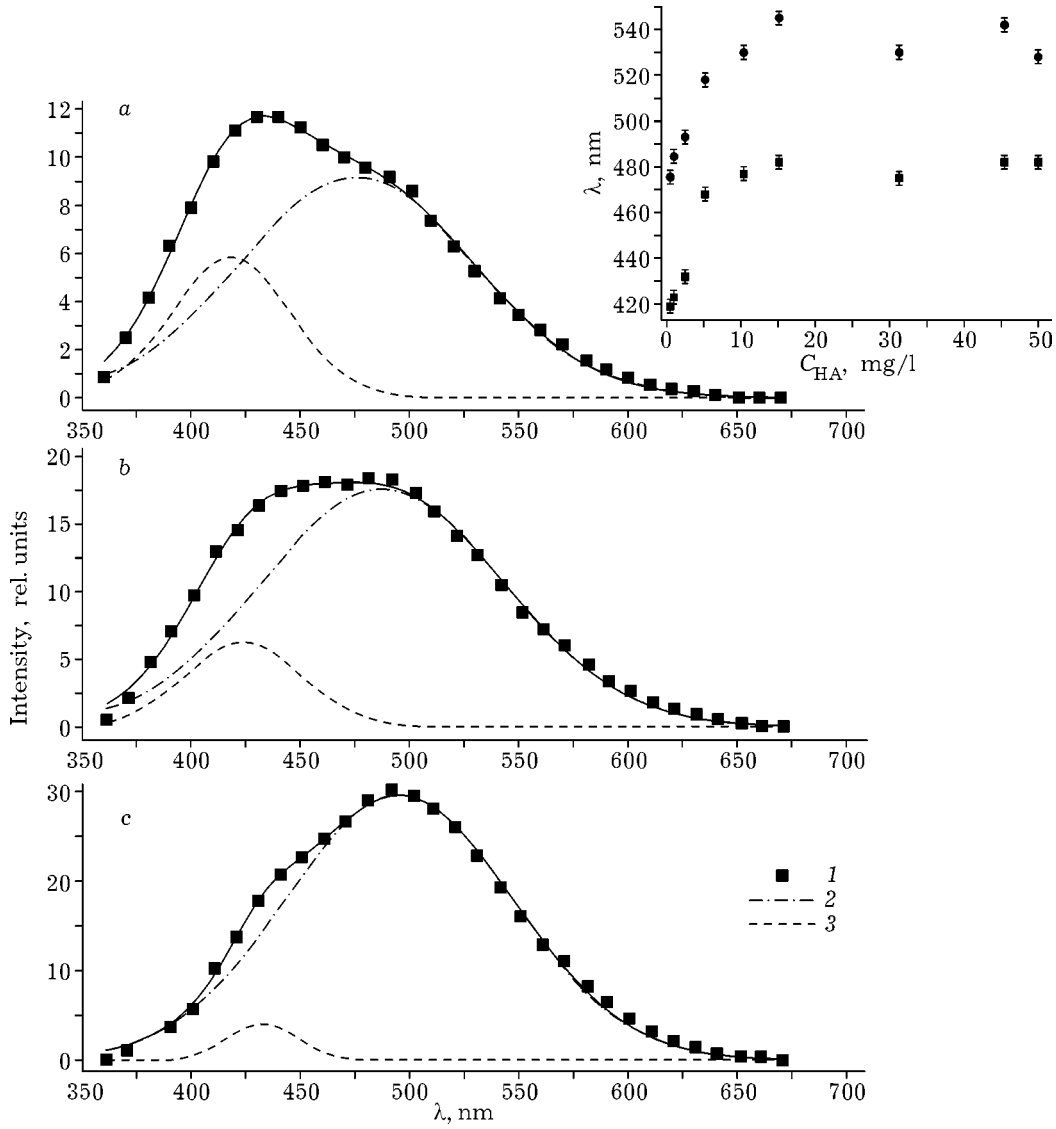

Fig. 3. Concentration dependencies of the components of fluorescence spectra shown in Fig. 2: *a* – C_{HA} = 0.5 mg/l; *b* – C_{HA} = 1 mg/l; *c* – C_{HA} = 2.5 mg/l; *1* – experiment; *2, 3* – the first and the second components, respectively; continuous line – a sum of the two components. Insert: Concentration dependencies of the positions of maxima of the components of fluorescence spectra: ■ – the first component; · – the second component.

tions differing in mass have different chemical nature. This difference involves an increase in the aromaticity of molecules in HA fractions increases with a decrease in fraction mass. A similar conclusion follows from the results of [20] showing that the fluorescence spectrum of a lighter HA fraction is in a longer-wavelength part of the spectrum. Our data confirm the results of [20]: M_1 value at which a plateau is observed increases with a decrease in fraction mass, that is, the fluorescence spectra of light fractions exhibit a batochromic shift with respect to the spectra of the heavier fractions.

The dependence of $\Delta M_1 = M_1' - M_1''$ (M_1', M_1'' are the first moments for C_{HA} equal to 50 and 5.0 mg/l, respectively) on the mean molecular mass of HA is shown in Fig. 5. It follows from this dependence that changes in M_1 decrease with an increase in HA fraction mass.

Possible nature of M_1 changes

The main experimental result is that an increase in HA concentration is accompanied with an increase in M_1. Interpretation of the observed dependencies is possible *a priori* within the fol-

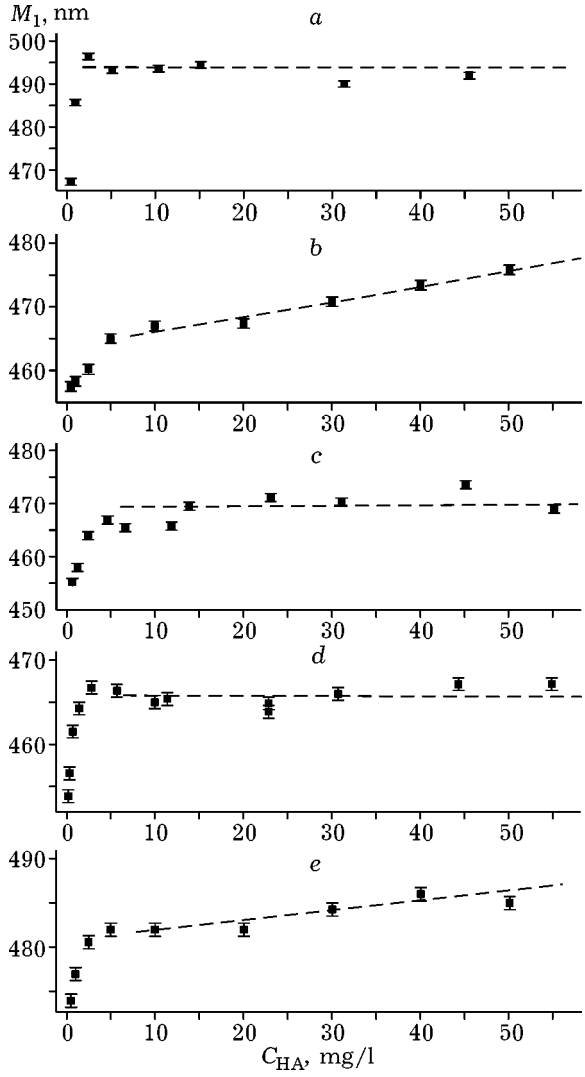

Fig. 4. Concentration dependencies of the first moments of fluorescence spectra (M_1) of different HA fractions: *a* – fraction C + D, *b* – fraction B + C, *c* – fraction B; *d* – fraction A; *e* – ungraded HA.

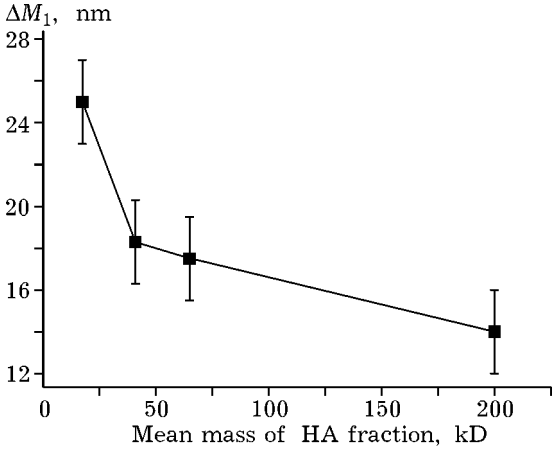

Fig. 5. Dependence of ΔM_1 on mean mass of HA macromolecules.

lowing hypotheses: a) the formation of excimers (exciplexes); b) the formation of rigid spherical colloids; c) formation of associates. Let us consider each of these hypotheses.

Formation of excimers. Indeed, the occurrence of these molecular formations (a complex composed of electronically excited and non-excited molecules) is able to explain the batochromic shift of fluorescence spectra with an increase in HA concentration: the fluorescence spectra of excimers are always observed at longer wavelengths than the spectra of the initial monomeric mollecules [45, 46]. However, the necessary condition for excimer formation is close HA fluorescence lifetime t_{fl} (t_{fl} is ~10^{-8} s [23, 42]) and the time of excimer formation t_{form}. The time of excimer formation is the value defines as $t_{form} \sim (K_{bim} C_{HA})^{-1}$, for HA concentration equal to 1 mg/l and mean HA molecular mass equal to ~50 kD and K_{bim} ~ 10^{-10} $M^{-1}s^{-1}$ this time is ~5 10^{-3} s, that is, several orders of magnitude longer than the fluorescence lifetime. So, the $t_{fl} \sim t_{form}$ condition is not fulfilled, so the hypothesis of the posisble excimer (exciplex) formation is unacceptable.

The formation of rigid spherical colloids as a result of twisting of the monomers. As it was indicated in the Introduction section, the possibility of the formation of spherical colloids with an increase in HA concentration was previously discussed in [2–5]. Within these notions, the occurrence of a plateau on the curve of M_1 dependence on HA concentration can be explained only by an increase in the number of spherical colloids (for the case when the shapes of spherical colloids are independent of concentration), while an increase in M_1 can be explained if the shape of spherical colloids depends on concentration. However, if, basing on the hypothesis of spherical colloid formation, we try to estimate quantitatively the distances at which HA molecules will interact with each other, a noticeable contradiction with the modern knowledge of distances at which intermolecular interactions become possible arises. Indeed, substantial changes in M_1 with an increase in HA concentration occurs as early as within the concentration range up to 1 mg/l. For these concentrations, mean distance R between the centres of the nearest HA macromolecules is about 400 nm. (The fraction of the lightest mass

C + D was taken to estimate R; HA concentration was 1 mg/l, mean molecular mass was 15 kD, mean mass of one atom was 10 D, and the distance R between two nearest neighbours for the concentration of macromolecules equal to C was estimated suing equation $R = 1.28C^{-1/3}$ [47].) This distance is two orders of magnitude larger than the size of macromolecule itself (~4 ´ 4 nm, if we consider it to be flat and accept that the distance between atoms is ~0.1 nm). There is no generally accepted opinion on the physical mechanism, which allows one to describe the interaction between two molecules in polar media at such a distance. So, we conclude that the hypothesis of the formation of rigid spherical colloids as a result of monomer twisting cannot explain the observed concentration dependencies of M_1.

The formation of associates can occur due to the aggregation of monomeric HA macromolecules. Within this hypothesis, changes in M_1 (see Fig. 4, for fractions B + C and ungraded) can be explained if we accept that the wavelength of the fluorescence of the associate (dimer, trimer, *etc.*) differs from the wavelength of monomer fluorescence. Indeed, in this case M_1 would change within the entire HA concentration range investigated because the ratio of the concentrations of associates differing in stoichiometric composition would depend on the total concentration of molecules. The occurrence of a plateau on the curve of M_1 dependence on concentration for these fractions (A, B and C + D) can be explained by the fact that the parameters of fluorescing groups do not undergo any changes after the formation of dimers, due to the structural features of HA macromolecule. So, due to the absence of principal contradictions in explaining the concentration dependencies of M_1, we are to prefer the hypothesis of associate formation. Below we consider possible specific physical reasons explaining the dependence of M_1 on HA concentration.

Nature of the shift of HA fluorescence spectrum with an increase in concentration

Interpreting the concentration dependence of the first moment of HA fluorescence spectra we assume that aggregation of the monomers takes place with an increase in HA concentration as a result of hydrophobic interactions. The possibility of such a process was previously mentioned in [14, 15, 25, 26]. The structure of HA associate is as follows: the fraction of polar fragments in direct contact with water and other polar groups of HA becomes larger than in the monomer, and the associate gets clearly exhibited micelle-like properties in comparison with a monomer. The measure with micelle-likeness may be, for example, the fraction of non-polar fragments of HA in direct contact with the solvent, or the size of pseudo-micellar microdomain [29].

Changes in M_1 value in the HA fluorescence spectra during HA association can be connected with two factors: 1) conformation changes of the fluorescing groups, and 2) changes in the polarity of microsurroundings of the fluorescing groups.

Conformational changes of fluorescing groups can be connected with the fact that, due to association process, the initial state of the structure of macromolecule is insignificantly deformed because of redistribution of the electron density inside the molecule. Physical factors causing these changes during association can be dipole-dipole interaction, electrostatic interaction and the presence of H-bond, as it is the case with aqueous solutions of proteins [48]. Redistribution of the electron density in HA molecule can lead either to changes of the angles between covalent bonds or to small changes of he distances between the atoms (for example, as a result of changes in the energy of one or several H-bonds during aggregation).

At present, it is difficult to prove that these structural changes lead to the batochromic shift of HA fluorescence band. *A priori*, it is equally probable that, as a result of association, redistribution of the electron density on chromohore groups can lead either to the batochromic or to hypsochromic shift of HA fluorescence spectra. In order to make a final conclusion, it is necessary to have irrefragable supplementary information about the chemical structure of fluorescing groups, their specific positions in the structural framework of HA macromolecule, about types of interactions and changes of these parameters inside one molecule accompanying changes in HA microstructure, *etc.* So, one of the possible physical reasons leading to

the batochromic shift can indeed be a change in the structure of fluorescing groups. This factor may take place independently of whether the polarity of the microsurroundings of a fluorescing group changes or not.

Effect of changes in polarity of microsurroundings of a fluorescing group on M_1 value. Microsurroundings include the nearest neighbours that can affect the fluorescing group due to the intermolecular interactions (dispersion forces and H-bonds). As a result of association, the fluorescing groups can turn to be in the surroundings different from those characteristic of the monomer microsurroundings. The possibility of this situation can easily be imagined if we take into account the fact that the structure of HA is able to form numerous chelate compounds with Mg^{2+}, Ca^{2+}, Fe^{2+} ions, *etc.* [49], so the fluorescing group can fall within changed microsurroundings. Another reason leading to changes in the microsurroundings of a fluorescing group as a result of association is undoubtedly hydrophobic interactions which would help straightening of polar and non-polar fragments, due to amphiphility of HA macromolecule, that is, micelle-like conformations will be formed. As a result, the fluorescing group, for example that situated in a polar part of the molecule, may fall within even more polar microsurroundings, while a fluorescing group from non-polar part may fall within a more non-polar one.

The polarity of microsurroundings (medium) affects the position of fluorescence spectrum [32, 33]. This is due to the fact that the levels of energy of the ground and excited states between which the radiational transition occurs survive different shifts depending on the polarity of surroundings. The position of fluorescence spectrum depending on the polarity of the medium is determined by the type of electron transition: with an increase in the polarity of the surroundings of a fluorescing group, a batochromic shift is observed for $\pi-\pi^*$ type and a hypsochromic one for $n-\pi^*$ type [32, 33]. (It should also be noted that specific donor-acceptor, electrostatic interactions and the formation of H-bonds of a fluorescing group of one molecule with a polar group of another molecule will cause batochromic shifts of HA fluorescence spectra for the $\pi-\pi^*$ type and hypsochromic shift for the $n-\pi^*$ type [32].) Specific indications for a type of fluorescing groups determining the fluorescence of HA macromolecules are absent from literature. There are only general considerations that the fluorescence of HA in the visible region can be due to either $\pi-\pi^*$ or $n-\pi^*$ types of electron transitions [16, 17].

So, within the model of associate formation, the character of observed concentration dependencies of M_1 is determined by two factors: conformation changes in the structure of humic acids and changes in the polarity of micro-surroundings of a fluorescing group. However, their simultaneous existence does not allow one to make unambiguous conclusions about the character of specific structural changes in aqueous solutions of HA. For example, if changes in HA fluorescence intensity were determined only by changes in the polarity of micro-surroundings, in case if the chosen model of HA aggregation is correct (and taking into account the fact that the quantum yields of fluorescence of fluorescing groups of $\pi-\pi^*$ type are much higher than those of $n-\pi^*$ type [32, 33]), observation of the batochromic shift of fluorescence spectra with an increase in HA concentration would allow us to make a conclusion that the major part of fluorescing groups in HA structure is in the polar surroundings. Correspondingly, the fluorescence spectrum of the former will exhibit batochromic shifts, and the latter – hypsochromic ones. Since experimentally we observe an overall change in the fluorescence spectra determined by changes in the state of all the fluorescing groups, the overall change will be due to the component of higher intensity. This component is that surviving the batochromic shift, that is, a component determined by fluorescing groups in the polar part of HA molecule. However, such an important conclusion cannot be considered to be quite correct because the initial prerequisite did not take into account the possible effect of the structural factor. In reality, the effect of each of the factors can be opposite; as both occur simultaneously, the effect of each of them may be veiled or become imperceptible. Because of this, a noticeable batochromic shift of the fluorescence spectrum within the concentration range 0–5 mg/l for all the fractions can be only one of the possible cases of

the effect of concentration changes on HA fluorescence spectra (for example, only conformation changes). Another partial case (M_1 is independent of HA concentration for $C_{HA} > 10$ mg/l) is realized for ungraded HA sample and for B + C fraction. In this case, for these samples, in addition to the previously proposed interpretation of the fact of M_1 independence of concentration, also another explanation is possible: the overall action of both factors is absent from this concentration range.

It may be assumed that in the case when the factor of HA structural changes with an increase in concentration will be prevailing in affecting the fluorescence spectrum, hypsochromic shifts will be observed as well. Hypsochromic shifts are also possible in the cases when fluorescing groups are mainly in the non-polar part of HA and the effect of HA structural deformation on the fluorescing group is small. Finally, a situation is possible when the dependence of M_1 on HA concentration will be non-monotonous: for example, if the effect of the first factor is positive and decreases with an increase in concentration, while the effect of the second one is negative and increases with an increase in concentration.

Due to the uncertainly of the effect of indicated factors on HA fluorescence spectra, an unambiguous interpretation of the data shown in Fig. 5 seems impossible even within the framework of the single model of association. For example, one of the possible explanations of larger changes of M_1 for small-mass fractions in comparison with larger ones (assuming the spectrum is determined by $p-p^*$ type of electron transitions, and the fluorescing group is in a polar microdomain) can be the following. During the formation of associates, small HA molecules undergo either more substantial structural changes of the macromolecule itself leading to the batochromic shift of fluorescence spectrum, or larger increase in the polarity of microsurroundings of the initial state of fluorescing group, or both factors at the same time. Another possible explanation is that the structural changes do not occur with an increase in concentration, only the microsurroundings of the fluorescing group change (for example, as a result of the formation of sandwich (plate-like) structures), which undergoes large changes for the fractions of small mass.

The data obtained can be interpreted from the traditional point of view on HA structure as a charged polymer, and from the point of view of the supramolecular model according to which HA structure is composed of relatively small molecules stabilized by hydrophobic forces. We cannot prefer one model or another on the basis of the results of investigation of the concentration dependence of fluorescence spectra of HA fractions.

CONCLUSIONS

The spectroscopic data obtained in the present work and the successive conclusions concerning structural changes in of HA macromolecule depending on concentration do not contradict known conclusions that an increase in concentration is accompanied by association, and its efficiency depends on the mass of the initial monomer [2, 29. 50]. The occurrence of the concentration dependence of the structure of HA macromolecule means that one should carefully use the common method of obtaining the information about binding constants of HA with different pollutants on the basis of experiments on quenching their fluorescence by HA macromolecules [33–35]. In these experiments, the structure of quencher molecule (HA) for each concentration is individual; therefore, actually quenching is carried out by the molecules different in their structures. This circumstance may bring uncontrollable errors into thus obtained binding constants. One cannot exclude that in some experiments this was the reason explaining the absence of linear dependencies in the experiments aimed at obtaining Stern–Volmer constants. So, in order to obtain reliable results, it is necessary to use preliminary information about the concentration dependence of HA macrostructure.

It should be noted that it is difficult to obtain the information of this kind on the basis of fluorescence data alone, because HA fluorescence spectra are multiparametric functions in which the arguments are the size of HA monomer, mobility of the links of HA macrostructure, polydispersity of HA sample itself, *etc.*, and for aqueous solutions – pH and ionic force. Finally, it should be noted that it is in-

correct to use the opinion that M_1 is proportional only to the degree of aromaticity in the molecule to interpret the data on the concentration dependence as it is accepted for simple molecules [22].

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