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DETERMINATION OF THE ISOSBESTIC POINT IN THE ABSORPTION SPECTRA OF DNA–ETHIDIUM BROMIDE COMPLEXES

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We propose a procedure for mathematical treatment of the spectra of complexes between ligands and DNA, which allows us to determine the position of the isosbestic point with high precision. As a specific example, we consider the absorption spectra of ethidium bromide complexes titrated against a DNA solution. As a result of using the method, besides the main type of binding we have identified the appearance of additional types of binding between the ethidium bromide and DNA.

Keywords: *DNA, DNA–ligand complexes, isosbestic point, absorption spectrum.*

Introduction. In studying interaction between different low molecular weight compounds and DNA, on the absorption spectra we observe isosbestic points, which are determined graphically and used to determine the fraction of ligand molecules bound to DNA [1, 2]. Such points are characterized by the same light absorption for different forms of the compound under study. In the case of DNA–ligand complexes, the presence of such a point is evidence that in the indicated region of variations in the concentration, an equilibrium exists between the two forms (free and bound) of the ligand molecules. The method is usually used in the case when bound and free ligand molecules have shifted absorption bands [3–5]. Due to the importance of the isosbestic point for various investigations, a considerable number of papers have been devoted to study of its behavior and methods for its determination (see, for example, [6–10]).

The procedure proposed in this paper allows us to use the isosbestic point to describe the interaction between the ligand and DNA, and to identify the existence of different types of binding between this ligand and the macromolecule. As an example, we consider the absorption spectra of a solution of ethidium bromide (EB) as it is titrated against a DNA solution [11, 12].

The Experiment. We used calf-thymus DNA and special purity ethidium bromide (Sigma-Aldrich, USA). None of the preparations were subjected to additional purification. The concentrations of the original DNA and ethidium bromide solutions were determined spectrophotometrically for the following extinction coefficients: $\varepsilon_{260} = 6600\ M^{-1}\cdot cm^{-1}$ for calf-thymus DNA [13] and $\varepsilon_{480} = 5800\ M^{-1}\cdot cm^{-1}$ for ethidium bromide [14]. The solutions were prepared according to [15].

Ethidium bromide was titrated against the DNA solution on a modified PYE UNICAM-SP8-100 spectrophotometer (UK). Control, measurement, and processing of the data obtained was carried out using a personal computer connected to the spectrophotometer and controlled by a program written in the LabVIEW environment. The spectral measurements were made in hermetically sealed quartz cuvets with optical path length 1 cm. The absorption spectra of the complexes were obtained in the wavelength interval $400 \le \lambda \le 600$ nm, in which there was no absorption by DNA. The absorption maximum for the original ethidium bromide solution (without the DNA) corresponds to $\lambda = 480$ nm. The original concentration of the ethidium bromide solution was 13.6 μg/mL. For titration, we used a DNA solution of concentration 1250 μg/mL. During the titration, the DNA solution was added to the ethidium bromide solution. The original volume of the EB working solution was 2.5 mL. The final volume of the DNA solution added to the EB solution was 0.6 mL. The DNA solution was added to the EB solution in such a way that during titration, the EB concentration in the working solution remained unchanged (did not decrease). To do this, 40 μL of EB with original concentration 36.5 μg/mL was added to the original DNA solution used for the titration. As a result, no dilution of the EB occurred in the working solution. During titration, the relative DNA concentration varied from 0 to 10. In each step of the titration, 15 μL of the DNA+EB mixture was added to the working solution.

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Calculation of the Position of the Isosbestic Point and Determination of the Minimum DNA Concentration Causing It to Shift. Let there be N titration spectra $A_i(\lambda)$ numbered $1 \le i \le N$ (Fig. 1). The spectrum numbered $i = 1$ corresponds to the original solution of unbound ethidium bromide, and larger values of i correspond to higher DNA concentrations. Let us pick out the group of spectra numbered $1 \le i \le k$, where k can be found in the interval $1 \le k \le N$, and let us calculate the average spectrum for it:

$$\overline{A}_k(\lambda) = k^{-1} \sum_{i=1}^{k} A_i(\lambda) . \quad (1)$$

Let us find the mean square deviation of the spectra in this group from its average spectrum:

$$\sigma_k(\lambda) = \left\{ k^{-1} \sum_{i=1}^{k} [A_i(\lambda) - \overline{A}_k(\lambda)]^2 \right\}^{1/2}, \quad \sigma_1(\lambda) \equiv 0 , \quad (2)$$

and also the mean square deviation for all N spectra:

$$\sigma(\lambda) = \left\{ N^{-1} \sum_{i=1}^{N} [A_i(\lambda) - \overline{A}_N(\lambda)]^2 \right\}^{1/2} . \quad (3)$$

The position of the minimum of the function $\sigma(\lambda)$ corresponds to the approximate position of the isosbestic point λ_{IP} (Fig. 2).

For the determined value of λ_{IP}, we calculate the N values $\sigma_k(\lambda_{IP})$ for $k = 1, ..., N$ (Fig. 3). Using this dependence, we find the interval of numbers for the spectra $k_1 \le i \le k_2$, characterized by a small value of $\sigma_k(\lambda_{IP})$, which should be used for exact determination of the position of the isosbestic point, and also the minimum DNA concentration for which deviation of the spectra from the isosbestic point occurs.

Results and Discussion. The proposed procedure for finding the isosbestic point and studying its properties was tested using the example of binding between ethidium bromide and DNA. Figure 1 shows the experimental absorption spectra for EB and its complexes with DNA (for clarity, we do not show all the spectra). As the DNA concentration increases, the intensity at the maxima of the spectra decreases and they are shifted toward longer wavelengths. In Fig. 1, we see the isosbestic point located at $\lambda \approx 510$ nm. Starting from certain ligand–DNA ratios, the absorption spectra of EB deviate from that point ([DNA][EB] > 6). The presence of an isosbestic point indicates that in solution, ethidium bromide is found in two spectrally distinct forms: free and bound. The shift of the spectra with high numbers from the isosbestic point is a consequence of the fact that a different binding mechanism appears for low degrees of coverage of the DNA by the EB molecules. Such deviations are also observed by other authors who, like us, used heterogeneous mammalian DNA for the investigation [16].

Based on the data in Fig. 1, using formula (3) we calculated the dependence $\sigma(\lambda)$ (Fig. 2). The minimum observed at $\lambda = 510$ nm corresponds to the approximate position of the isosbestic point ($\lambda_{IP} = 510$ nm). In order to refine its position, we plotted the dependence of $\sigma_k(\lambda_{IP})$ on k (Fig. 3), where the point with coordinates (1;0) corresponds to the value of $\sigma_1(\lambda_{IP}) = 0$, obtained using spectrum 1 in Fig. 1; the second point is $\sigma_2(\lambda_{IP})$, obtained using spectra 1 and 2, etc. The large spread in the initial points is a consequence of the small samples. The points in the middle part within experimental uncertainty fit on a horizontal line. This region ($k = 4$–26) corresponds to spectra slightly deviating from the isosbestic point. For the spectra numbered from $k_1 = 4$ to $k_2 = 26$, the calculation is repeated, i.e., $\sigma_{k1,k2}(\lambda)$ is calculated:

$$\overline{A}_{k1,k2}(\lambda) = (k_2 - k_1 + 1)^{-1} \sum_{i=k_1}^{k_2} A_i(\lambda) , \quad (4)$$

$$\sigma_{k1,k2}(\lambda) = \left\{ (k_2 - k_1 + 1)^{-1} \sum_{i=k_1}^{k_2} [A_i(\lambda) - \overline{A}_{k1,k2}(\lambda)]^2 \right\}^{1/2} . \quad (5)$$

Calculations using formulas (4) and (5) confirmed the initially determined position of the isosbestic point $\lambda_{IP} = 510$ nm. According to Fig. 3, for high DNA concentrations ($k > 26$), when the degree of coverage of the double helix by EB molecules is low, deviation from the isosbestic point begins. The deviation is connected with the appearance of another type

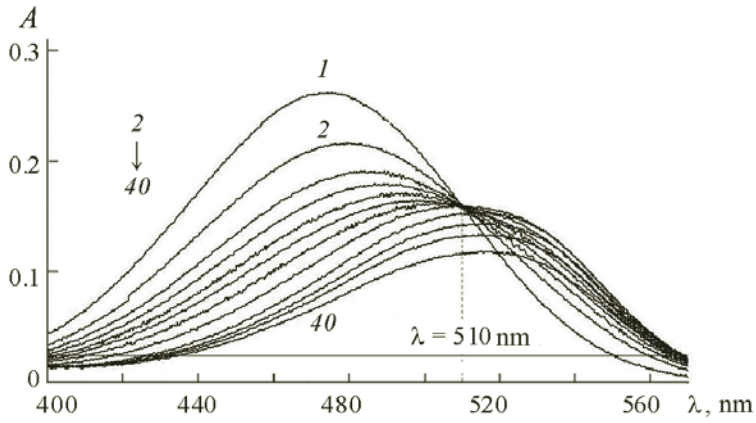

Fig. 1. Absorption spectra for ethidium bromide (1) and its complexes with DNA (2–40).

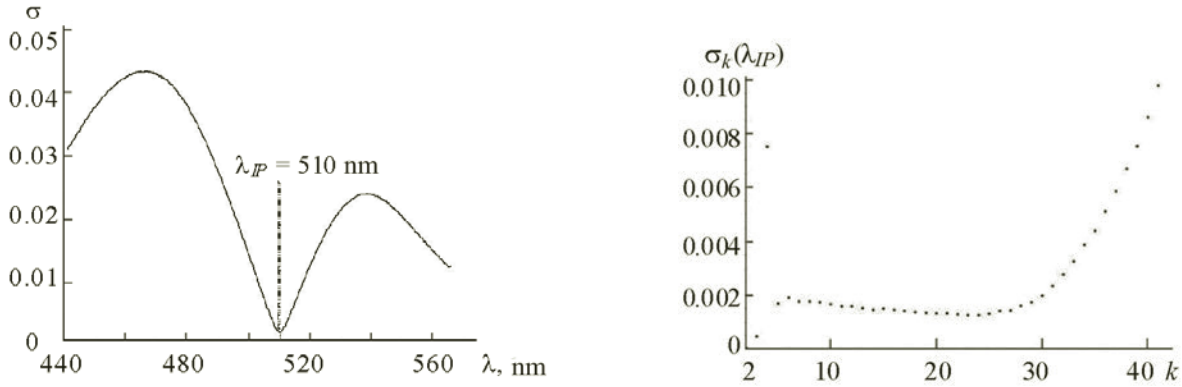

Fig. 2. Mean-square deviation of the absorption vs. wavelength σ(λ), calculated using formula (3), for all the spectra ($k = N = 40$).

Fig. 3. Mean-square deviation in the isosbestic point $\sigma_k(\lambda_{IP})$ vs. the number of the spectra k used to calculate σ_k from formula (2); for each k, the numbers of the spectra used for the calculations lie within the interval $1 \le i \le k$.

of binding between EB and DNA. Thus the results obtained are evidence that for ethidium bromide, at least two types of adsorption centers exist. In Fig. 3, they correspond to the regions $k = 1$–26 and $k > 26$.

Conclusions. Using the proposed procedure for determining the position of the isosbestic point and estimating the DNA concentration region from which deviation of the spectra from it begins, we consider the spectra for titration of an ethidium bromide solution against a DNA solution. Based on the method used, the behavior of the spectra in the region of the isosbestic point helps us identify an additional type of binding between ethidium bromide and DNA, besides the main binding mechanism.

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