MOLECULAR BIOPHYSICS

Investigation of complexation of ethidium bromide with DNA by the method of Raman spectroscopy

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The investigation of complexation features of ethidium bromide (EB) with calf thymus DNA at the high and low ratios of biopolymer/ligand molar concentrations (P/D) was carried out using Raman spectroscopy and VIS-spectrophotometry. It was shown that EB binds to DNA with formation of two types of complexes: intercalation and exterior binding. The analysis of Raman spectra revealed that the amino groups of EB form the hydrogen bonds with acceptor atom groups of DNA in both types of complexes. A low extent of filling DNA structure by the ligand (P/D = 20) does not change the DNA B-form, while a high extent (P/D = 3) results in the conformation B-A transition.

Keywords: ethidium bromide; DNA; complex; Raman spectroscopy, spectrophotometry.

Introduction. The study on molecular mechanisms of interaction of biologically active compounds with nucleic acids is still a very actual issue. The formation of various types of complexes between intercalating ligands and DNA can lead to changes in structure and functional activity of nucleic acids

During many years ethidium bromide (EB) was used as a classical intercalator. However, only at the last years the theoretical study on melting curves of DNA-EB complexes [1], experimental investigations of titration curves and adsorption isotherms by differential pulse voltammetry (DPV) [2] showed a possibility of arranging in EB-DNA system not only intercalating complexes but at certain conditions a formation of complexes of external binding type .

Earlier the infrared spectroscopic investigations of EB-DNA films at various relative humidities (RH) [3] showed that the formation of EB-DNA complex was accompanied by decreasing hydration of DNA sugar-phosphate backbone. The intercalation of the ligand occurs in GC-sites at the minor DNA groove. However, such investigations were performed only at P/D (molar ratio of DNA to EB) equal 4.0, that did not allow to find the differences in spectral parameters of EB-DNA complexes depending on different concentrations of both intercalator and DNA. As it was shown by the Raman spectroscopy method in [4], the EB intercalation into DNA at low P/D 6 induced a structural transition from B- to A- form of DNA. There is still no answer to the question whether the spectral changes of EB-DNA mixtures are connected with the formation of several types of complexes or with the conformational alterations of DNA matrix at various level of its filling by EB.

In this work we investigated the binding of EB to calf thymus DNA (ctDNA) at high and low values of P/D using Raman spectroscopy and VIS-spectrophotometry. The aim of these studies was elucidation of the molecular mechanisms of EB binding to DNA and detection of the B-A structural transition in DNA molecule as a function of EB binding level.

Materials and methods. We used ethidium bromide from "Fluka" (Switzerland) and calf thymus DNA from "Serva" (Germany) without additional purification. All solutions were prepared in phosphate buffer solution ($2.5\cdot10^{-2}$ M KH_2PO_4; $2.5\cdot10^{-2}$ M Na_2HPO_4) at pH 6,86.

The concentrations of EB and DNA were determined using $_{480} = 5860\ M^{-1}\cdot cm^{-1}$ [5] and $_{260} = 6400\ M^{-1}\cdot cm^{-1}$ molar extinction coefficients, correspondingly. All DNA-EB mixtures were prepared at the constant ligand concentration ($C_{EB} = 1{,}1\cdot10^{-4}$ M) and various P/D values. The DNA films were prepared by water vaporization from 10^{-3} M DNA solution at 4^oC. To obtain DNA films in A- or B- form we controlled the relative humidity (RH) in the hermetically closed cells by using saturated solutions of NaCl (76% RH) and K_2SO_4 (96% RH), respectively [6-8].

Spectrophotometric measurements were carried out in the thermostatic quartz cells with optical path length of 10 mm in spectrophotometer Specord M 40 (Germany).

The Raman spectra of EB and DNA-EB mixtures were recorded using DILOR Z-16 spectrometer (France) with double-monochromator. The spectrometer calibration was based on the spectral frequencies of CCl_4 [9]. Here, we used argon laser with excitation line = 514,5 nm which is located in the absorption band of EB, that provides obtaining the pre-resonance Raman spectra of EB. The solutions of free EB and its mixtures with DNA, and DNA films were put in quartz cells (10 mm). To avoid saturation effects and decomposition of the samples, the energy of laser did not exceed 25 mW. The spectral split width was 4,5 cm^{-1}, and the scanning speed was 30 cm^{-1}/min. The accuracy of reproducibility of Raman frequencies was ±2 cm^{-1}. The standard computer software was used for the treatment of Raman spectra All spectra were recorded at room temperature.

Results and discussion. Figure 1 shows the structural formula of EB molecule and absorption spectra of EB-DNA mixtures at different P/D values.

The absorption maximum of free EB is observed at =480 nm (Figure 1b, spectrum 1). As can be seen, this spectrum is shifted to long-wave region upon EB binding to DNA ($_{max}$ = 524 nm). This process is also accompanied by decreasing of the ligand absorption (Figure 1b, spectra 2 and 3). The DNA-EB mixtures were homogeneous at P/D>8 for all EB concentrations. However, at lower P/D values, we observed separation of DNA-EB mixtures into two optically clear fractions.

Figure 1b shows that at 3 P/D 20 the spectra of DNA-EB mixtures are similar and differ very little in the 400 – 550 nm wavelengths interval. Therefore, to characterize the different types of binding of EB to DNA, it was necessary to use more sensitive methods of investigations. We have chosen the Raman spectroscopy which allows to determine the groups of atoms of the EB molecule participating in complex formation with DNA at high and low P/D values. Using this method we also could observed the conformation changes of the DNA molecule in the complexes.

It is known that EB in aqueous solutions can be in monomer and dimer forms depending on its concentration [10]. We recorded the Raman spectra of EB at various concentrations to determine the spectral differences between monomer and dimer forms. Table 1 demonstrates the the Raman frequencies of EB as polycrystalline sample and in buffer solutions at C_{EB} 10^{-2} M and C_{EB} 10^{-4} 10^{-5} M. The assignments of frequencies for the ethidium bromide atom groups have been made on the basis of literature data [11 - 14]. Thus, the bands at =1372 - 1377 cm^{-1} are attributed to the valency symmetrical vibrations of conjugated C–C and C–N bonds of the phenanthridinium ring. The bands at =1411 – 1417 cm^{-1} are assigned to the phenanthridinium ring breathing vibration [12].

From the Table 1 follows that the 1372 cm-1 band assigned to vibrations of C–C and C–N groups of the phenanthridinium ring is observed at the high EB concentration (C_{EB}~10^{-2} M)., There are equal parts of monomer and dimmer forms of the ligand at this EB

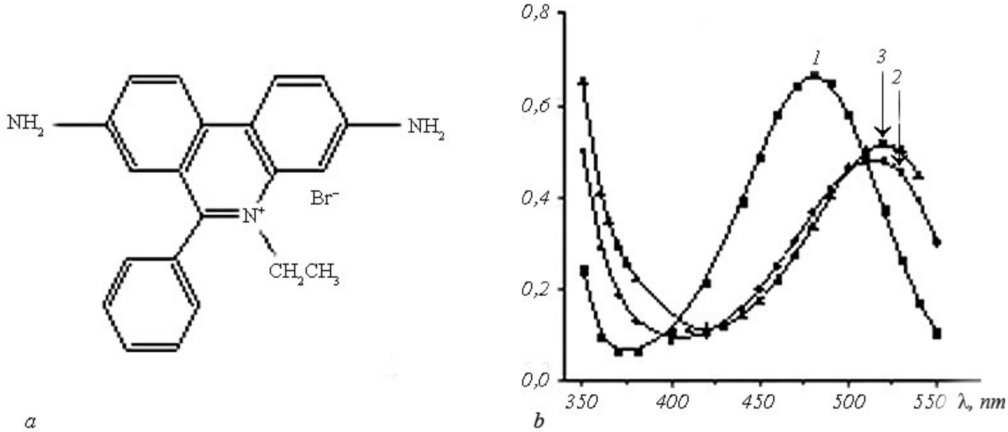

Fig. 1. Structural formula of EB (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) (*a*) and absorption spectra of EB-ctDNA mixtures (*b*) at P/D = 0 (*1*); P/D = 3,3 (*2*) and P/D = 20 (*3*). C_{EB} = 1,1 10^{-4} M.

Table 1.
Raman frequencies and their assignments to crystal sample and solutions of EB at two concentrations (C).

Assignment	Crystal EB, cm^{-1}	EB (C 10^{-2} M), cm^{-1}	EB (C 10^{-4} M), cm^{-1}
Phenanthridinium ring (C-C, C-N)	1354 1375	1350 1372	1351 1377
CH3, deformation	1389	1394	1389
Phenanthridinium ring breathing	1412	1417	1411
CH2, deformation	–	1442	1434
CH3, deformation	1454	1462	1452
Phenyl ring	1605	1605	1602
NH2, deformation	1626	1627	1626

concentration [15]. At the lower EB concentrations (C_{EB} 10^{-4} 10^{-5} M), a monomeric form of EB prevails [15] and the maximum of corresponding Raman spectra is shifted to higher frequency range =1377 cm^{-1} We think that the red shift of this band to =1372 cm^{-1} is associated with interaction of the ligand aromatic rings in dimers.

Figure 2 shows the pre-resonance Raman spectra of free EB and its mixture with DNA. The corresponding Raman frequencies and their assignments are given in Table 2 for the free EB and bound to DNA. Since the concentrations of the free ligand are very low in DNA-EB mixtures at the DNA concentrations considered here, we could attribute the differences in Raman spectra of EB at two P/D values to different types of the ligand binding to DNA. As it is evident from Raman spectrum of EB-DNA complex at P/D=20 (Fig. 2, spectrum 3), the vibration band of the phenanthridinium ring with the maximum at =1377 cm^{-1} is red-shifted by 5 cm^{-1} in comparison with the band of free EB. A similar low-frequency shift is also observed for EB dimers that seems to be evidence of the interaction of aromatic EB ring with nitrogen atoms of DNA bases. Therefore, this shift of the band of phenanthridinium ring could be used as a criterion of the EB chromophore intercalation between the base pairs of DNA. A similar effect was observed in Raman spectra of some aromatic components intercalated into DNA [16].

The Raman spectrum of the complex at P/D=3 (Figure 2, spectrum 2) shows that the maximum of the free EB band at =1377 cm^{-1} is not shifted to low-frequency region in the presence of DNA. We assumed that in the DNA-EB mixtures at low P/D values a prevailed amount is formed of complexes where EB is externally bound to DNA and the concentration of intercalated ligand in mixtures is decreased and less manifested in the total Raman spectra.

High-frequency shift is observed for CH_2- and CH_3-deformational vibrations at the formation of both types of complexes EB with DNA (Figure 2, spectra 2 and 3, band assignments see in Table 2). Table 1 demonstrates that these vibrations are sensitive also to monomer-dimer transition. However, the interpretation of such results requires additional investigations.

The other band of phenanthridinium ring with maximum at =1411 cm^{-1} (Figure 2, spectrum 1) attributed to ring breathing is shifted to higher

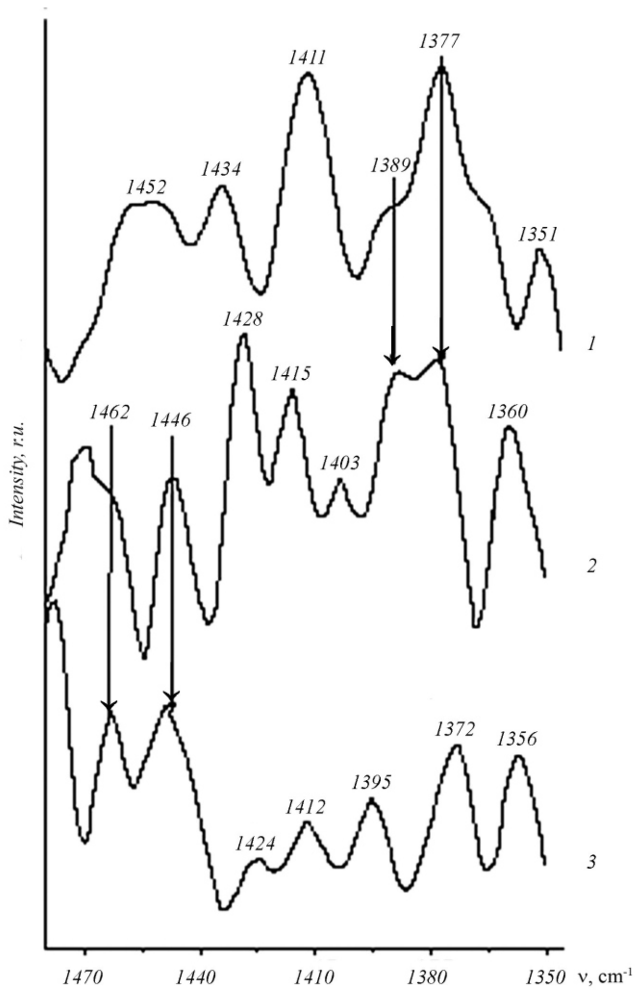

Fig. 2. Pre-resonance Raman spectra of EB (*1*) and DNA-EB complexes at P/D = 3 (*2*) and P/D = 20 (*3*) in 1340-1470 cm^{-1} spectral region. C_{EB} = 1,1·10^{-4} M.

frequency =1415 cm^{-1} in solution with P/D=3. A similar effect was observed in the work [12] upon the substitution of hydrogen atom of NH_2- group of EB in the 2-amine-4-chloro-6-methylpyrimidine ring. The position of this band does not change at the P/D=20. These results can also testify to different types of complexes formed in DNA-EB system at various P/D values. However, an unambiguous conclusion requires more detailed study like in a case of CH_2- and CH_3-groups.

Figure 3 shows the pre-resonance Raman spectra of free EB and DNA-EB mixtures at the same as in Figure 2 P/D values in the interval of frequencies =1600-1660 cm^{-1}. The main contribution to this

Table 2.
Raman frequencies and their assignments for EB in complex with DNA in the 1350-1465 cm^{-1} spectral range.

Assignment	EB (C 10^{-2} M), cm^{-1}	DNA–EB	
		P/D = 3, cm^{-1}	P/D = 20, cm^{-1}
Phenanthridinium ring (C-C, C-N)	1351 1377	1360 1377	1356 1372
CH3, deformation	1389	1388	1395
Phenanthridinium ring breathing	1411	1415	1412
CH2, deformation	1434	1446	1447
CH3, deformation	1452	1462	1462

spectral region is made by the deformational vibrations of NH_2-groups [11, 13] and the vibrations of EB phenyl ring of [12, 13]. The corresponding Raman bands and assignments for EB are given in Table 3.

The spectra 2 and 3 (Figure 3) and the data given in Table 3 demonstrate that the formation of two types of complexes at both P/D=3 and P/D=20 is accompanied by high-frequency shift by 5 cm^{-1} of the deformational vibration band of NH2-group in phenanthridinium chromophore [12]. This fact signifies the formation of hydrogen bonds between NH_2-groups of EB and acceptor groups of DNA.

Taking into account the model of EB phenanthridinium chromophore intercalation into DNA [14], N1 atom of cytosine and N9 atom of guanine can be the acceptor groups of the opposite DNA chains. On the other hand, a possibility of formation of H-bonds between NH_2-groups of EB intercalated in DNA GC-sites and the atoms O4' and O5' of the guanine deoxyriboses of opposite chains was shown by the method of molecular docking [17]. Such model is confirmed by the X-ray study of the EB-d(CpG) complex in which H-bonds between NH_2-groups of EB and oxygen atom O5' of deoxyriboses have been found [18].

Thus, the high-frequency shift of band of deformational vibration of NH_2-groups of EB at high and low P/D values confirms the formation of hydrogen bonds between these groups and DNA like in case of both EB intercalated into DNA or bound

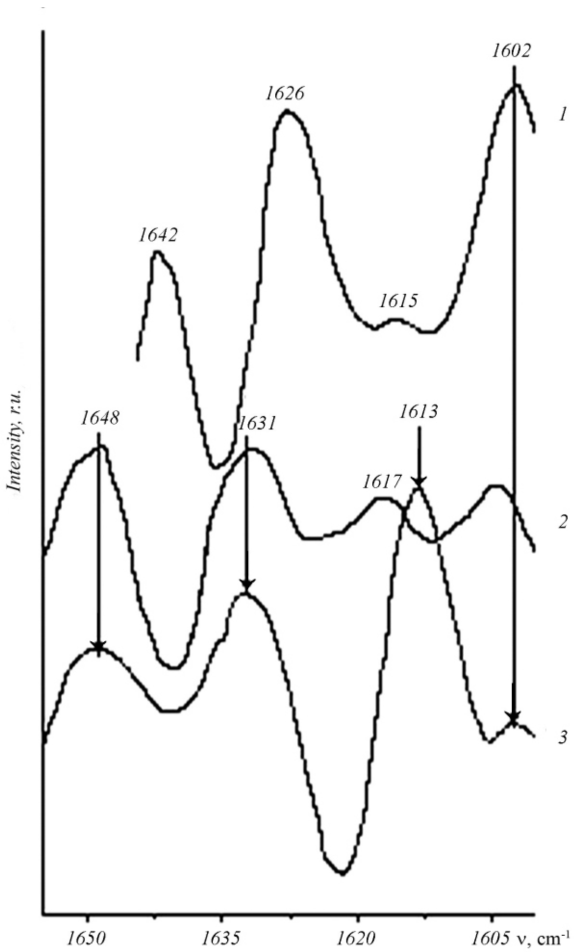

Fig. 3. Pre-resonance Raman spectra of EB (*1*) and its complexes with ctDNA at P/D = 3 (*2*) and P/D = 20 (*3*) in 1600 -1660 cm^{-1} spectral region. C_{EB} = $1{,}1\cdot10^{-4}$ M.

externally. All these hydrogen bonds which appear parallel with stacking interactions of the EB aromatic rings and nitrogen bases (cytosine and guanine) stabilize additionally the DNA-EB complex resulting in increase of its thermostability [19].

To establish the DNA structure in complexes with EB at low and high P/D values, we recorded the Raman spectra of complexes in range of the DNA marker bands [6, 20 – 22].

Figure 4 shows spectra of free EB and its mixtures with DNA in the region of frequencies = 750–850 cm^{-1} at two P/D values for DNA in both A- and B-forms. A very low intensity of EB Raman bands in this spectral region in comparison with DNA allows us to neglect the contribution of ligand vibrations in

Table 3.
Raman frequencies and their assignments for EB complexed with DNA in the 1600-1650 cm^{-1} spectral region.

Assignment	EB (C » 10-2 M), cm^{-1}	DNA–EB	
		P/D = 3, cm^{-1}	P/D = 20, cm^{-1}
Phenyl ring	1602	1603	1602
Phenyl ring(C-C)	1615	1617	1613
NH2, deformation	1626	1631	1631

spectrum of complex and to take into account the DNA vibrations only.

One can see from spectrum 2 (Figure 4) that the complex formation at high-density filling of DNA matrix (P/D=3) leads to the appearance of bands at = 780 cm^{-1} and 807 cm^{-1}. It is known [6, 20-22] that these bands are typical markers for A-form of DNA (Figure 4, spectrum 5). Therefore, our studies confirm the conclusions made earlier in [3, 4,13] about transition of DNA from B- to A- like conformation at increasing of filling density of DNA matrix by EB molecules. In the Raman spectra at P/D=20 we have observed the bands at = 834, 796 and 781 cm^{-1} (Figure 4, spectrum 3), which are the marker bands of B-form of DNA [6, 20-22]. Hence, the DNA bound to EB at high P/D values (P/D 20) was in B-conformation.

Thus, the complexation of EB with DNA at low P/D 3 is apparently accompanied by displacement of EB dye from intercalation sites and structural transition of DNA from B- to A-like form. In this case, the minor groove of DNA becomes wider and shallower that could lead to external binding of the ligand.

Conclusions. The investigation of complex formation between EB and ctDNA by Raman spectroscopy allowed to conclude that in DNA-EB solutions at high P/D values and low concentrations of the ligand, stabilization of the complex occurs not only due to the interaction of intercalating chromophore with DNA, but due to the formation of H-bounds between amino groups of EB and oxygen atoms of deoxiribose (O4’ and O5’) as well. In the last case, DNA retains in B-form. At low P/D values, the DNA-EB complex formation is accompanied by structural transition of DNA from B- to A-like

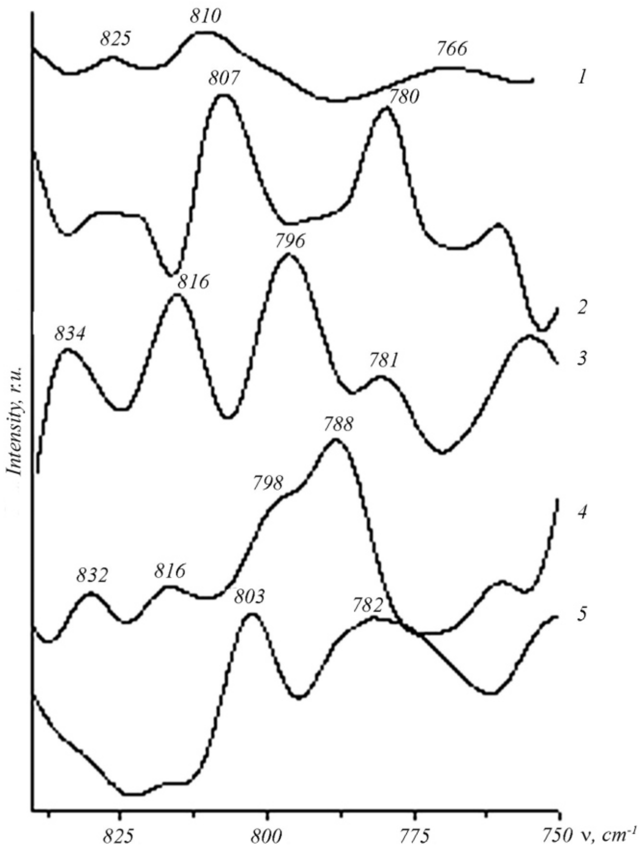

Fig. 4. Pre-resonance Raman spectra of EB (*1*), EB-DNA complexes at P/D=3 (*2*) and P/D=20 (*3*) and spectra of B-DNA (*4*) and A-DNA (*5)* films at relative humidities of 96% and 76% , respectively. $C_{EB} = 1{,}1 \cdot 10^{-4}$ M.

conformation, and amino groups of EB interact with phosphate groups of sugar-phosphate backbone of polynucleotide matrix stabilizing external "fix" of EB chromophore on the surface of DNA molecule.

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Исследование комплексообразования бромистого этидия с ДНК методом спектроскопии комбинационного рассеяния света

Резюме

Методами спектроскопии комбинационного рассеяния (КР) света и спектрофотометрии в видимой области исследованы особенности комплексообразования бромистого этидия (ЭБ) с тимусной ДНК при высоких и низких соотношениях концентраций биополимер/лиганд (P/D). Показано, что взаимодействие ЭБ с ДНК осуществляется двумя способами: по типу интеркаляции и за счет внешнего связывания. Из анализа спектров КР комплексов следует, что аминогруппы ЭБ образуют водородные связи с атомами кислорода O4' и O5' ДНК в обоих типах комплексов. Низкая степень заполнения ДНК лигандом (P/D = 20) не изменяет В-формы ДНК, а высокая (P/D = 3) – приводит к конформационному В–А-переходу.

Ключевые слова: бромистый этидий, ДНК, комплекс, спектроскопия комбинационного рассеяния, спектрофотометрия.

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Дослідження комплексоутворення бромистого етидію з ДНК методом спектроскопії комбінаційного розсіяння світла

Резюме

Методами спектроскопії комбінаційного розсіяння (КР) світла та спектрофотометрії у видимій області досліджено особливості комплексоутворення бромистого етидію (ЕБ) з тимусною ДНК при високих та низьких співвідношеннях концентрацій біополімер/лiганд (P/D). Показано, що взаємодія ЕБ з ДНК відбувається двома способами: за типом інтеркаляції та за рахунок зовнішнього зв'язування. З аналізу спектрів КР комплексів випливає, що аміногрупи ЕБ формують водневі зв'язки з атомами кисню O4' і O5' ДНК в обох типах комплексів. Низький ступінь заповнення ДНК лігандом (P/D = 20) не змінює В-форми ДНК, а високий (P/D = 3) – призводить до конформаційного В–А-переходу.

Ключові слова: бромистий етидій, ДНК, комплекс, спектроскопія комбінаційного розсіяння, спектрофотометрія.

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