SPECTRAL PROPERTIES OF 8-BROMOETHENO DERIVATIVES OF ADENINE NUCLEOTIDES

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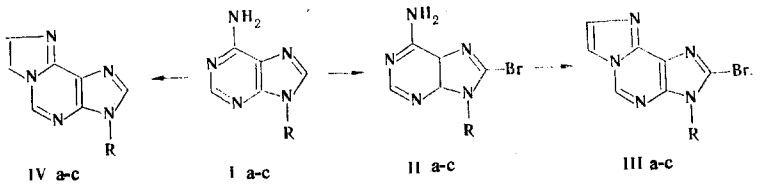
Imidazo[2,1-i]purine derivatives, viz., etheno-AMP, etheno-ADP, and etheno-ATP, as well as their 8-bromo derivatives, were synthesized by means of α,β-dibromoethyl acetate. The spectral properties of the compounds obtained were studied. It was observed that, with respect to the character of their electronic and fluorescence spectra, the brominated analogs of ethenoadenine nucleotides do not differ from the unbrominated compounds. The introduction of a bromine atom into the imidazo[2,1-i]purine system leads to a decrease (by a factor of 24) in the fluorescence quantum yield and to the appearance of phosphorescence [$\tau = (2 \pm 1) \cdot 10^{-2}$ sec]. The conformations of the compounds in aqueous solutions were studied.

Purine nucleotides participate in a large number of enzyme reactions. Their analogs are therefore of interest for the solution of a number of enzymological problems. Among the purine nucleotides, the ε derivatives, which were first synthesized in 1971-1972 by means of chloroacetaldehyde [1, 2], have proved to be a godsend. These compounds, which differ little from natural nucleotides with respect to their structure, have high affinities for the active centers of enzymes and have high fluorescence quantum yields. They have therefore found extensive application [3, 4]. The spectral properties and conformations of ε-adenine nucleotides have been described [5-7]. The synthesis of a number of analogs of ethenoadenine nucleotides, including 8-bromoetheno derivatives, is also known [8-10]. However, nothing is known regarding the spectral properties of the brominated analogs of ethenoadenine nucleotides.

To study the spectral properties of these compounds we accomplished the synthesis of 3-β-D-ribofuranosylimidazo[2,1-i]purine 5'-mono-, di-, and triphosphates (IVa-c, ε-AMP, ε-ADP, and ε-ATP, respectively), as well as their corresponding 8-bromo derivatives IIIa-c, via the scheme



IV a-c I a-c II a-c III a-c

I—IV a R = 3-β-D-ribofuranosyl-5'-phosphate; b R = 3-β-D-ribofuranosyl-5'-diphosphate; c R = 3-β-D-ribofuranosyl 5'-triphosphate

The synthesis of etheno derivatives IVa-c was carried out as described in [11] by the action of α,β-dibromoethyl acetate at pH 4.5-5.5 on the corresponding adenine nucleotides Ia-c. To obtain the corresponding 8-bromoadenine nucleotides IIIa-c we carried out the bromination of adenine nucleotides Ia-c as described in [12], and the resulting 8-bromo derivatives IIa-c were subjected to condensation with α,β-dibromoethyl acetate by the method in [11]. Compounds IIIa-c were characterized by the results of elementary analysis and by their chromatographic and electrophoretic mobilities.

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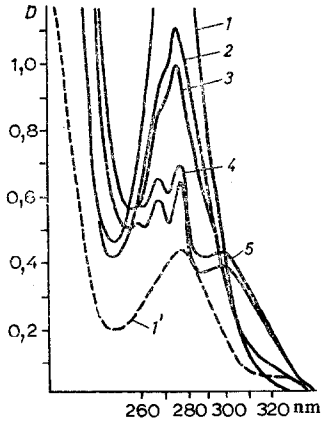

Fig. 1

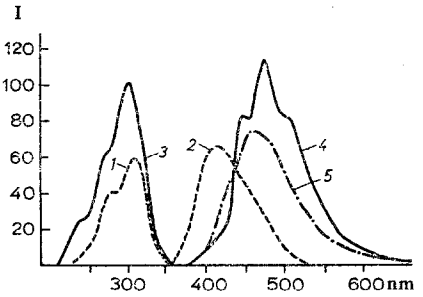

Fig. 2

Fig. 1. Electronic spectra of Br-ε-ATP at various pH values: 1) pH 2.0; 2) pH 3.0; 3) pH 4.0; 4) pH 4.5; 5) pH 10. For curves 1-5 the concentration of the nucleotide was $1 \cdot 10^{-4}$ M, whereas for curve 1' the concentration of the nucleotide was $3.3 \cdot 10^{-5}$ M. The spectra were recorded in a universal buffer solution containing 0.04 M phosphoric, acetic, and boric acids.

Fig. 2. Fluorescence and phosphorescence excitation and emission spectra of Br-ε-ATP: 1) excitation spectrum, recording of the fluorescence at 420 nm; 2) fluorescence spectrum, excitation at 312 nm; 3) excitation spectrum, recording of phosphorescence at 470 nm; 4) phosphorescence spectrum at pH 8.0, excitation at 310 nm; 5) the same as for curve 4 but at pH 6.0. The fluorescence spectra were recorded at a nucleotide concentration of $1 \cdot 10^{-4}$ M in a 0.02 M phosphate buffer, pH 7.4. The phosphorescence spectra were recorded at 77°K in a 0.04 M universal buffer solution.

As observed for **ethenoadenine** nucleotides IVa-c [5, 13, 14], IIIa-c have identical absorption and fluorescence spectra. In the preent paper we will therefore discuss their spectral properties in the case of only one compound, viz., 8-bromoetheno-ATP (IIIc).

The electronic spectra of nucleotides IIIa-c depend markedly on the degree of protonation of the Br-ε-adenine heteroring (Fig. 1). The resolved structure of the spectrum appears at pH > 4. As in the case of IVa-c [5], four absorption maxima are observed: λ_{max} ($\varepsilon \cdot 10^{-3}$): 258 (5.0), 265 (6.0), 275 (6.0), and 304 nm (3.1). The electronic spectrum does not depend on the pH at pH 7-10. It is evidently characteristic for the unprotonated structure.

The fluorescence excitation and emission spectra are presented in Fig. 2. The excitation spectrum contains a band at 300-320 nm that is not overlapped with the absorption band of the aromatic **amino acids** of the proteins. This fact may be useful in the investigation of the fluorescence spectra of IIIa-c in the **presence** of proteins. One unresolved band with a maximum at 415-420 nm is observed in the fluorescence spectrum. The fluorescence quantum yield of IIIc determined by comparison with the quantum yield of IVc is 0.025 ± 0.005, which is smaller by a factor of 24 than in the case of IVc. The decrease in the quantum yield is due to the presence of a heavy atom in the fluorescing ring [15].

The fluorescence quantum yield depends markedly on the pH (Fig. 3), and the fluorescence pK_a value is 4.1 ± 0.1. As in the case of IVc [13, 14], an unprotonated structure is evidently responsible for the fluorescence of IIIc.

Divalent metal ions form complexes with natural nucleotides; the metal ion is bonded to the triphosphate part and is in direct proximity to the adenine part of the molecule [16-18]. To ascertain the degree to which the structure of IIIc in solution differs from the structure of the natural nucleotide we studied the effect of a number of ions of divalent metals on the fluorescence of the adenine part of the molecule during complexing.

Divalent Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} ions caused quenching of the fluorescence of triphosphate IIIc. With respect to their degree of quenching of the fluorescence these ions are arranged in the order $\mathbf{Mn^{2+}} < Zn^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+}$. Alkali earth metal ions such as Mg^{2+}, Ca^{2+}, and Cd^{2+} had no effect on the fluorescence of IIIc.

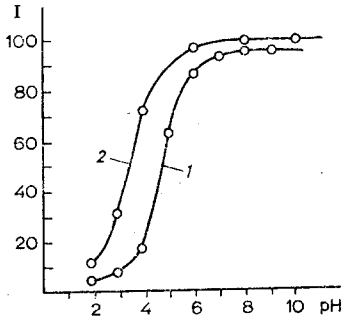

Fig. 3

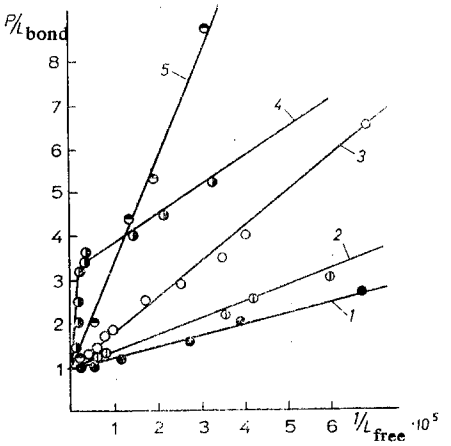

Fig. 4

Fig. 3. Dependence of the fluorescence and phosphorescence emission of Br-ε-ATP on the pH: 1) fluorescence, excitation at 312 nm, fluorescence at 420 nm; 2) phosphorescence, excitation at 310 nm, phosphorescence at 470 nm. The nucleotide concentration was $1 \cdot 10^{-4}$ M. The fluorescence was recorded at 293°K, while the phosphorescence was recorded at 77°K.

Fig. 4. Dependence of P/L_{bond} on $1/L_{free}$ for a number of divalent metal ions: 1) Cu; 2) Ni; 3) Co; 4) Zn^{2+}; 5) Mn.

To determine the bonding constants and the number of bonding centers we used the equation [19]

$$\frac{P}{L_{bond}} = \frac{1}{n} \cdot \frac{1}{K_{bond}} \cdot \frac{1}{L_{free}} + \frac{1}{n},$$

where L_{bond} is the concentration of bonded metal ions, L_{free} is the concentration of free metal ions, P is the starting concentration of IIIc, n is the number of bonding center, and K_{bond} is the bonding constant; L_{bond} was determined from the expression $(I_o - I)P/(I_o - I_k)$, where I_o is the intensity of the fluorescence of the nucleotide in the absence of metal ions, I is the maximum value of the fluorescence at a given metal ion concentration, and I_k is the maximum fluorescence, which depends on the nature of the added metal ion.

The dependence of P/L_{bond} on $1/L_{free}$ has linear character (Fig. 4) for the Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} ions. Since n = 1, the ratio of IIIc and the metal ion in the complex is unity. In the case of Zn^{2+} the dependence of P/L_{bond} on $1/L_{free}$ has a discontinuity. Approximately three bonding centers are observed at low L concentrations, whereas one bonding center is observed at high concentrations. The reason for this behavior of Zn^{2+} ions is not clear. The dissociation constants were determined from the slopes. The values obtained are presented in Fig. 5 and Table 1. A correlation between the pK_d and pK_a values is observed for these metals. The zinc ion constitutes an exception. The existence of an inflection on the line for the zinc ions is evidently the result of amphoteric properties of this ion. On the basis of the dependences obtained it may be assumed that the degree of complexing and quenching of the fluorescence of IIIc is due primarily to the acid–base properties of the metal ions.

Quenching of the fluoresence of IIIc by metal ions can be observed when these ions are in direct proximity to the heterocyclic part of the molecule. Quenching evidently occurs either due to direct protonation of the heteroring by a polarized water molecule situated, as previously assumed for ε-ATP [18], between the metal ion and the heterocyclic part of the molecule or due to a shift of the electron density from 8-Br-ε-adenine to the metal ion. As in the case of the ATP molecule, the IIIc molecule should therefore exist in a folded conformation in solution (Fig. 6).

In contrast to ε-ATP, the brominated ε-ATP derivative has fluorescence. The phosphorescence excitation and emission spectra are presented in Fig. 2. When the pH of the solution is shifted to the acidic region, the excitation maximum in the excitation spectrum is shifted to the short-wave region.

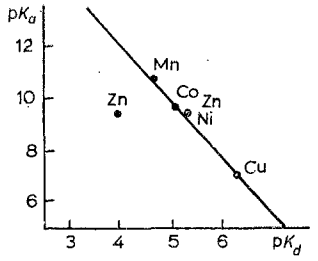

Fig. 5. Dependence of pK_d on pK_a for metal ions.

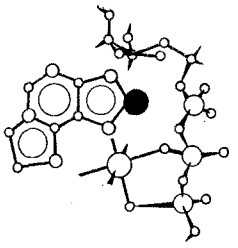

Fig. 6. Hypothetical structure of the Br-ε-ATP–divalent metal ion.

TABLE 1. Dissociation Constants of 8-Br-ε-ATP (IIIc) with Divalent Metal Ions[a]

Metal ion	pK_a	I_k, %	K_d in M	pK_d[b]	K_d[c] for ε-ATP in M
Mn^{++}	0,7	24,8	$2,26\times10^{-5}$	4,6	$8,3\times10^{-6}$
Zn^{++}	9,6	69,7	$4,6\times10^{-6}$	5,3	$5,9\times10^{-5}$
			$1,5\times10^{-4}$	3,9	
Co^{++}	9,6	96,2	$8,0\times10^{-6}$	5,1	$1,07\times10^{-5}$
Ni^{++}	9,4	97,0	$5,0\times10^{-6}$	5,3	—
Cu^{++}	7,53	98,0	$4,5\times10^{-6}$	5,34	$2,44\times10^{-6}$

[a]The I_k value is the maximum quenching by a given metal ion in percent, pK_a is the basicity constant of the metal ion calculated from the K_a values from [20], K_d is the effective dissociation constant of the [nucleotide–metal ion] complex, and $pK_a = -\log K_a$. [b]$pK_d = -\log K_d$. [c]See [3] for the determination of K_d.

A maximum at 470 nm with shoulders at 445 and 510 nm is observed in the phosphorescence spectrum at pH 6–7. On passing to the acidic region the fine structure of the spectrum is smoothed out, and the phosphorescence maximum is shifted to 460 nm. The dependence of the phosphorescence maximum is shifted to 460 nm. The dependence of the phosphorescence intensity on the pH is presented in Fig. 3, from which it is apparent that IIIc phosphoresces weakly in the acidic region. The maximum phosphorescence appears at pH 6-10. It might be assumed that a deprotonated structure of the heteroring is responsible for both the phosphorescence and the fluorescence. The pK_a of phosphorescence is 3.9 ± 0.2. The phosphorescence lifetime is $(2 \pm 1)\cdot10^{-2}$ sec. Like the fluorescence, the phosphorescence of IIIc is quenched by divalent metal ions. These data serve as an additional confirmation that IIIc exists in a folded conformation in solution.

Our study shows that the introduction of a heavy atom into the ε-ATP structure does not affect the excited electron levels and the overall structure of the ε-ATP molecule; however, it markedly decreases the fluorescence quantum yield and leads to the appearance of phosphorescence. The fact that the IIIc molecule has simultaneously both fluorescent and phosphorescent properties opens up prospects for the extensive application of Br-ε-adenine nucleotides in the study of the topography of substrate-bonding or regulator centers of enzymes simultaneously from both the fluorescence and the phosphorescence parameters of the modified nucleotide after affined introduction into the composition of an active or regulator center of a number of enzymes, as previously described for ε-ATP [21-23].

EXPERIMENTAL

The electronic spectra of the compounds were measured with a Specord spectrophotometer (East Germany), while the fluorescence and phosphorescence spectra were recorded with an Amin-co-Bowman spectrofluorimeter (USA). Thin-layer chromatography (TLC) was carried out on Silufol UV-254 plates in the following solvent systems: a) isobutyric acid–water–triethylamine (66:33:1); b) dioxane–water–ammonium hydroxide (6:4:1). Electrophoresis was carried out in a 0.5 M citrate buffer with pH 5.0 for 1 h on Whatman ZMM paper at 1200-1500 V and a current strength of 30-50 mA. The kinetics of quenching of phosphorescence were studied with the apparatus described in [24]; λ_{exc} = 337 nm, pulse energy = $3\cdot10^{-4}$ J with pulse time $\tau = 10^{-8}$

sec. In this research we used $MgCl_2$ obtained by reaction of spectrally pure MgO with distilled HCl. The $CaCl_2$, $CoCl_2$, $CdCl_2$, $MnCl_2$, $ZnCl_2$, $NiCl_2$, and $CuCl_2$ (chemically pure grade) were used without additional purification.

The synthesis of IIa-m was carried out by the method in [12]. The brominated nucleotides obtained were used for the synthesis of IVa-c without additional purification. α,β-Dibromoethyl acetate was synthesized by the method in [25].

8-Br-3β-D-Ribofuranosylimidazo[2,1-i]purine 5'-Monophosphate, 8-Br-3β-D-ribofuranosylimidazo[2,1-i]purine 5'-Diphosphate, and 8-Br-3β-D-Ribofuranosylimidazo[2,1-i]purine 5'-Triphosphate (IIIa-c). These compounds were obtained by reaction with α,β-dibromoethyl acetate as described in [11]. The course of the reaction was followed spectrophotometrically from the change in the fluorescence intensity. Compounds IIIa-c were purified by chromatography with a column (2 by 25 cm) packed with DEAE-Sephadex A-25. Compound IIIa was eluted with a linear gradient of a triethylammonium bicarbonate buffer with pH 7.5 (0-0.3 M), while IIIb and IIIc were eluted with a linear gradient of the same buffer (0-0.5 M). The fraction of the corresponding nucleotides was dried lyophilically. For complete removal of the triethylamine, a few millimeters [sic] of water were added to the dried preparation, ethanol was added, and the mixture was dried repeatedly. Nucleotides IIIa-c were converted to the sodium salts by dissolving in ethanol and precipitation with a 1% solution of $NaClO_4$ in acetone; the excess perchlorate was removed by washing the precipitate with acetone and diethyl ether.

Compound IIIa, with mp 180-185°C (dec.), was obtained in 95% yield. Found: C 37.0; H 3.2%. $C_{12}H_{12}O_7$. Calculated: C 34.6; H 3.6%. The product had R_f 0.52 (a) and 0.53 (b). Compound IIIb, with mp 187-195°C (dec.), was obtained in 90-92% yield. Found: C 29.4; H 3.3%. $C_{12}H_{13}O_{10}$. Calculated: C 29.0; H 3.2%. The product had R_f 0.48 (a) and 0.40 (b). Compound IIIc, with mp 195-202°C (dec.), was obtained in 85-87% yield. Found: C 23.5; H 3.2%. $C_{12}H_{14}O_{13}$. Calculated: C 23.6; H 3.0%. The product had R_f 0.3 (a) and 0.19 (b).

The synthesis of IVa-c and their purification were carried out as described above.

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