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DETERMINATION OF PROTONATION SITE IN 1,N^6-ETHENOADENOSINE RESIDUE IN AQUEOUS SOLUTION

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We have prepared hitherto unknown quaternized derivatives of εAdo[1], i.e., m^1εAdo$^+$ and m^9εAdo$^+$. These are the nontautomerizable model compounds for two possible forms of protonated εAdo, H^1εAdo$^+$ and H^9εAdo$^+$, respectively. A rough estimate of the [H^1εAdo$^+$]/[H^9εAdo$^+$] ratio is obtained by comparing the ultraviolet absorption spectra of protonated εAdo and the two nontautomerizable reference compounds. The results of this study will be valuable to resolve literature differences with regard to the fluorescence nature of protonated εAdo.

That the 1,N^6-ethenoadenosinium cation exists mainly in the N9-protonated form in the solid state has been established by X-ray crystallography for 7-ethyl 1,N^6-ethenoadenosine hydrochloride[2]. However, the question concerning the site of protonation in aqueous solution remains uncertain although it has been suggested by arguing from NMR data for εAdo and the protonated εAdo that N9 is the primary site of protonation in εAdo and presumably in the nucleotides as well[3-5]. Such conclusion, however, necessarily presuppose that protonation occurs exclusively at one site. At low pH, protonation might occur at either of at least two possible sites, N1 and N9, in the εAdo molecule because of its structural similarity to guanosine and 1-methyladenosine; an equilibrium might exist between the possible tautomeric forms[6], e.g., H^1εAdo$^+$ $\rightleftharpoons$ H^9εAdo$^+$.

To determine the percentage of H^1εAdo$^+$ to H^9εAdo$^+$, hitherto unknown N-methyl (quaternized) derivatives, m^1εAdo$^+$ and m^9εAdo$^+$, have been prepared by direct methylation[7]. With dimethylsulfate εAdo undergoes methylation to give a mixture(1:8) of m^1εAdo$^+$ and m^9εAdo$^+$. The reaction mixture was taken to dryness under reduced pressure at room temperature and the residue was first chromatographed on Dowex 1x2 (NH_4^+ form) to separate methylated products from other materials. A mixture of m^1εAdo$^+$

and $m^9\varepsilon Ado^+$ was then subjected to thin-layer chromatography on Eastman Chromatogram cellulose sheets using n-butanol/ethanol/0.05% acetic acid(80/10/25) as devoloping agent. The two separated spots were eluted with water and freeze-drying gave purified materials. The ultraviolet absorption spectra of $H^x\varepsilon Ado^+$ and their quaternary N1- and N9-methyl derivatives with fixed structures have been measured and found to be unaffected by the pH value over the range 1 to 7[7]. By comparing spectra, equilibrium constant for $H^1\varepsilon Ado^+ \rightleftharpoons H^9\varepsilon Ado^+$ has been estimated as follows: It is found that $m^1\varepsilon Ado^+$ absorbs radiation at a longer wavelength than $m^9\varepsilon Ado^+$(Fig. 1). The spectra of the two methylated forms are very different, so that the intensities of the long-wavelength bands can afford a measure of the tautomeric equilibrium if there are appreciable amounts of both forms, $H^1\varepsilon Ado^+$ and $H^9\varepsilon Ado^+$, present at equilibrium. Methyl groups, for the most part, exert only second-order effects on the intensity of allowed electronic transitions(ε>1000)[8,9], and accordingly the spectra of N1- and N9-methyl derivatives may be taken as the good models for the absorption characteristics of $H^1\varepsilon Ado^+$ and $H^9\varepsilon Ado^+$, respectively. On this assumption a rough estimate of the $[H^1\varepsilon Ado^+]/[H^9\varepsilon Ado^+]$ ratio in aqueous solution may be made using the extinction values at 280 to 310 nm range of the fixed model compounds for the protonated tautomers after correction for the small bathochromic effects due to substitution of a methyl group for a proton in $m^1\varepsilon Ado^+$ and $m^9\varepsilon Ado^+$ by 1 nm and 4 nm, respectively[10]; $[H^1\varepsilon Ado^+]/[H^9\varepsilon Ado^+] = 0.15 \pm 0.10$.

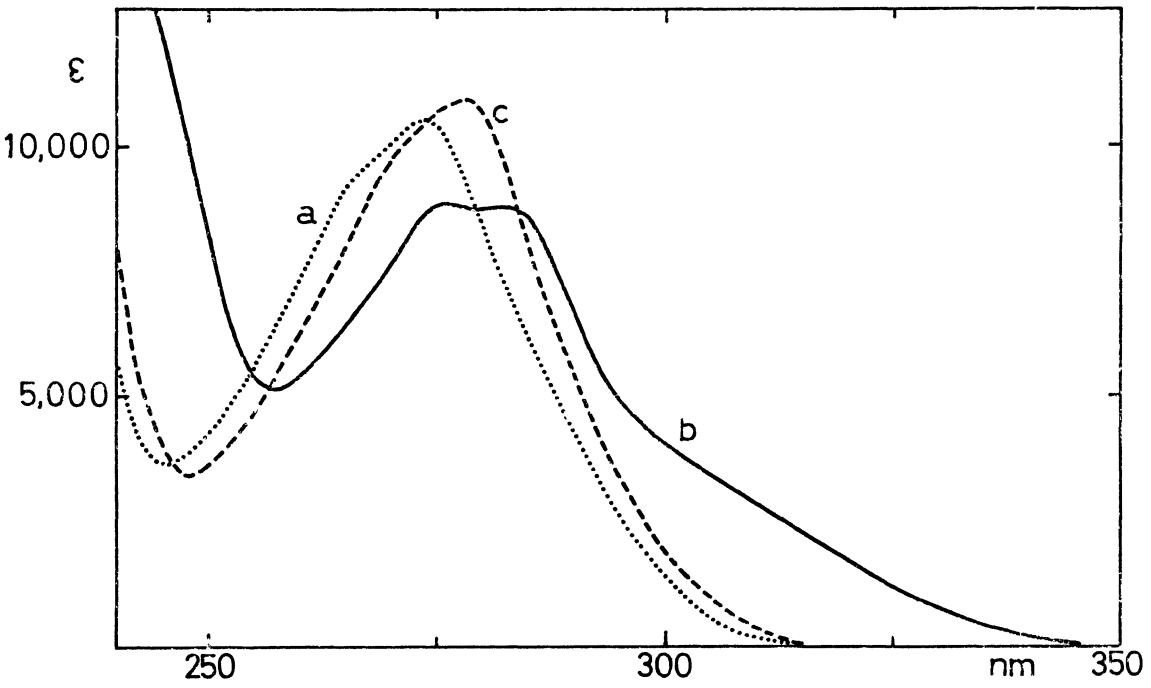
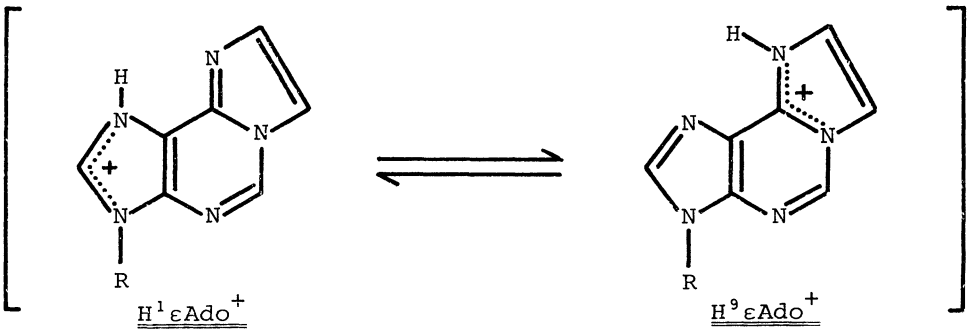


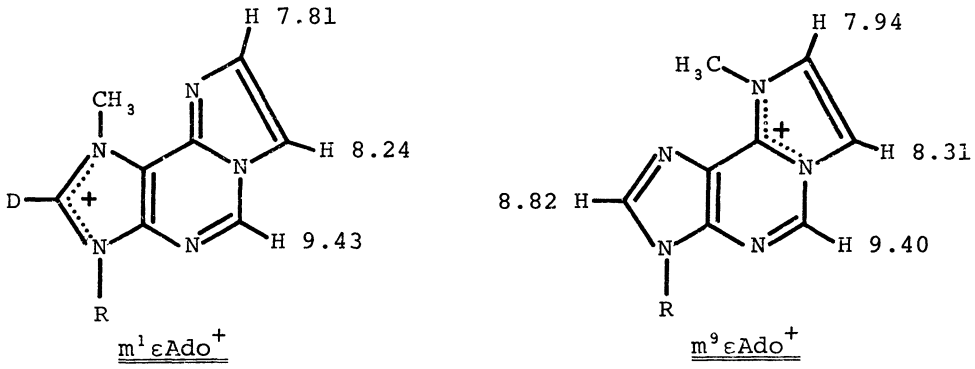
Fig. 1. Ultraviolet absorption spectra of: (a)·····, the cation $H^x\varepsilon Ado^+$(at pH 1); (b)———, $m^1\varepsilon Ado^+$(at pH 7); and (c)------, $m^9\varepsilon Ado^+$(at pH 7).

Supporting evidence in favor of $H^9\varepsilon Ado^+$ is also provided by the qualitative similarity between the ^1H NMR spectra of the protonated εAdo and $m^9\varepsilon Ado^+$ (Scheme I)[11]. The present results are even more convincing than the results described by Leonard et al.[3] and Penzer[4].

δ_{H2} 8.83
δ_{H5} 9.43
δ_{H7} 8.31
δ_{H8} 7.96

R = ribosyl





Scheme I (chemical shifts from DSS, in ppm)

A preliminary account of protonation and quaternization of εAdo given in this communication is particularly interesting in view of the extensive series of recent investigations tending to identify the species responsible for fluorescence of εAdo. Details on the preparation and purification of $m^1\varepsilon Ado^+$ and $m^9\varepsilon Ado^+$ and their fluorescence nature will be reported elsewhere[14].

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References and Notes

1) Abbreviations used: εAdo, 1,N^6-ethenoadenosine(or 3-β-D-ribofuranosylimidazo[2,1-i]purine); $H^1\varepsilon Ado^+$ and $H^9\varepsilon Ado^+$, a pair of tautomeric isomers protonated on N1 and N9 of εAdo, respectively (For the numbering system for εAdo, see ref. 3 or 4); $m^1\varepsilon Ado^+$ and $m^9\varepsilon Ado^+$, x-methyl-3-β-D-ribofuranosylimidazo[2,1-i]purinium cations where x is 1 and 9, respectively.

2) A.H.-J. Wang, L.G. Dammann, J.R. Barrio, and I.C. Paul, J. Am. Chem. Soc., 96, 1205(1974).

3) J.A. Secrist III, J.R. Barrio, N.J. Leonard, and G. Weber, Biochemistry, 11, 3499 (1972).

4) G.R. Penzer, Eur. J. Biochem., 34, 297 (1973).

5) R.D. Spencer, G. Weber, G.L. Tolman, J.R. Barrio, and N.J. Leonard, Eur. J. Biochem., 45, 425 (1974).
6) By analogy with the previous results of the protonation studies on adenosine[12], guanosine[12], and 3,N^4-ethenocytidine[13], it is highly unlikely that protonation occurs at the N4 atom to form H^4εAdo$^+$ or at the bridgehead nitrogen, N6.
7) The conditions for methylation must be carefully selected to prevent both loss of D-ribose by acid hydrolysis and rapid ring-opening of the products at alkaline pH.
8) S.F. Mason, J. Chem. Soc., 5010 (1957); A.R. Katritzky and J.M. Lagowski, in "Advances in Heterocyclic Chemistry" (Ed. A.R. Katritzky), Vol. 1, p.328 (1963).
9) N. Ogasawara, Y. Watanabe, and Y. Inoue, J. Am. Chem. Soc., 97, 6571 (1975).
10) On replacement of an N7-H in H^7Guo$^+$ by methyl group the absorption band 256nm is displaced to a longer wavelength by only 1 nm, while in going from H^6m^1Ado$^+$ to $H^6m^1_2{}^{,6}$Ado$^+$ the absorption band 257 nm is shifted bathochromically to 261 nm.
11) The assignment of the base protons of εAdo follows Secrist III et al.(1972)[3], where the assignment was carried out by selective deuteration, resulting in $\delta_{H5} > \delta_{H2} > \delta_{H7} > \delta_{H8}$.
12) V.A. Bloomfield, D.M. Crothers, and I. Tinoco, Jr., "Physical Chemistry of Nucleic Acids", p.77, Harper & Row, Publishers, New York (1974) and references therein.
13) J.R. Barrio, P.D. Sattsangi, B.A. Gruber, L.G. Dammann, and N.J. Leonard, J. Am. Chem. Soc., 98, 7408 (1976).
14) Y. Inoue, T. Kuramochi, and K. Imakubo, a manuscript in preparation.

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