DEMETHYLAMINATION OF N^6-METHYLDEOXYADENOSINE BY COMMERCIAL SPLEEN PHOSPHODIESTERASE PREPARATIONS*

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Received December 14,1973

SUMMARY: Commercial spleen phosphodiesterase preparations contain an activity (presumably enzymatic) that will deaminate adenosine, deoxyadenosine and demethylaminate N^6-methyldeoxyadenosine but not their 3'- or 5'-phosphates. The product obtained by the enzymic demethylamination of N^6-methyldeoxyadenosine was identified as deoxyinosine on the basis of its electrophoretic mobility and its ultraviolet spectrum. The presence of a deaminase activity in spleen phosphodiesterase preparations must be considered when using the enzyme in nucleotide sequence studies.

INTRODUCTION. Spleen phosphodiesterase is an exonuclease used extensively in the nucleotide sequence analysis of RNA (1) and DNA (1, 2). The DNA nucleotide sequence methylated by the specific modification methylase (Eco RI methylase) controlled by an fi^+ R-factor in Escherichia coli has been recently determined (3) and the methylated base was identified as N^6-methyladenine. In the above work commercial spleen phsophodiesterase was routinely used to hydrolyze oligonucleotides to nucleoside 3'-phosphates. When an oligonucleotide lacking a 3'-phosphate, e.g. a trinucleoside diphosphate is hydrolyzed, the digest also contains a free nucleoside originating from the 3'-terminus (NpN'pN'' Np + N'p + N''). Difficulties were encountered because no N^6-[^{3}H]methyldeoxyadenosine could be found in the hydrolysate whenever the methylated nucleoside was at the 3'-terminus of an oligonucleotide (position N''), although it could always be recovered as a 3'-phosphate from internal (position N'p) or 5'-terminal locations (position Np) of any oligonucleotide. No such difficulty occured when the same oligonucleotide was digested with snake venom phosphodiesterase. Since addition of excess non-radioactive N^6-methyldeoxyadenosine to the spleen phosphodiesterase digests resulted in good recovery of the N^6-[^{3}H]methyl-

*Supported by U.S. Public Health Service Grants CA 14026 and GM 14378, American Cancer Society Grant No. NP-112A, Cancer Research Funds of the University of California, and University of California MSC No. 30 Hampton Fund.

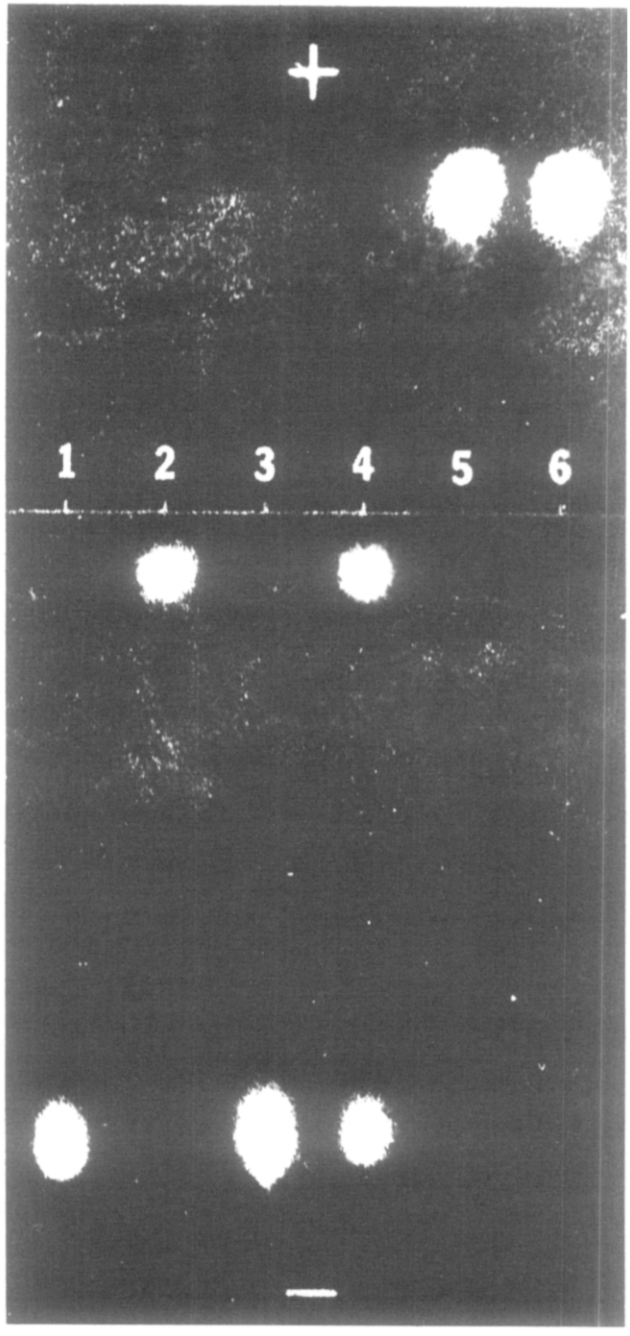
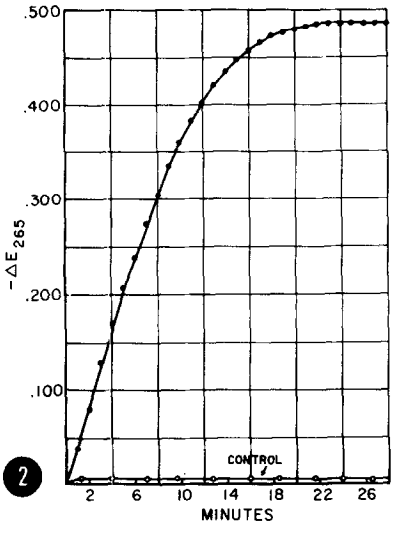


Fig. 1. Electrophoretic separation on Whatman No. 1 paper (two hours, 35 V/cm in a pyridine/acetic acid buffer, pH 3.5) of the products of digestion of deoxyadenosine and N^6-methyldeoxyadenosine with spleen phosphodiesterase (about 0.1 μ mole of a deoxynucleoside was digested in a capillary for two hours at 37° with about 30 μl of spleen phosphodiesterase (20 units/ml) in 50 mM Tris HCl buffer, pH 7.4, 20 mM $MgCl_2$). Position 1: Deoxyadenosine, 2: its digestion product, 3: N^6-methyldeoxyadenosine, 4: its digestion product, 5: deoxyadenosine 5'-phosphate, 6: its digestion product.



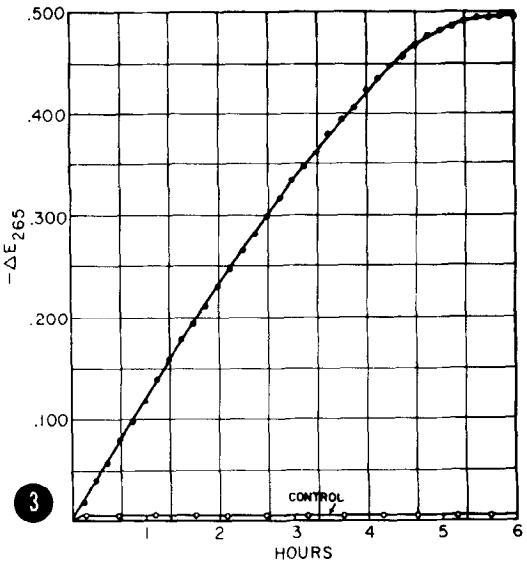


Fig. 2. Kinetics of deamination of deoxyadenosine at 25°, observed spectrophotometrically at 265 nm. The cuvette contained in a volume of 3 ml: 50 mM Tris HCl buffer (pH 7.4), 20 mM $MgCl_2$, 0.178 μ mole deoxyadenosine, and 0.15 unit of spleen phosphodiesterase. The control, incubation under the same conditions in the absence of spleen phosphodiesterase, is shown by the open circles. After 24 minutes, 99% of the deoxyadenosine was accounted for from the decrease of absorbance at 265 nm. The initial slope corresponds to a deamination of 0.016 μ mole of deoxyadenosine per min.

Fig. 3. Kinetics of demethylamination of N^6-methyldeoxyadenosine at 25°, observed spectrophotometrically at 265 nm. The cuvette contained in a volume of 3 ml: 50 mM Tris HCl buffer (pH 7.4), 20 mM $MgCl_2$, 0.151 μ mole of N^6-methyldeoxyadenosine, and 1.5 units of spleen phosphodiesterase. The control, incubation under the same conditions in the absence of spleen phosphodiesterase, is shown by the open circles. After 5.5 hours, 97% of the substrate was accounted for from the decrease of absorbance at 265 nm. The initial slope corresponds to a deamination of 5.7×10^{-4} μ moles per min.

deoxyadenosine, this led us to suspect that it must have been a contaiminating deaminase that was responsible for the results, and the N^6-methyldeoxyadenosine was one of its substrates. The present communication presents evidence that commercial preparations of spleen phosphodiesterase contain such a deaminase activity.

MATERIALS AND METHODS. N^6-Methyldeoxyadenosine was purchased from Terra-Marine Bioresearch (La Jolla, California). It contained some deoxyadenosine which was removed chromatographically on Whatman No. 1 paper impregnated with $(NH_4)_2SO_4$ and using a solvent system composed of 80 volumes of 95% ethanol and 20 volumes of water (4). Spleen phosphodiesterase (3.1.4.1)

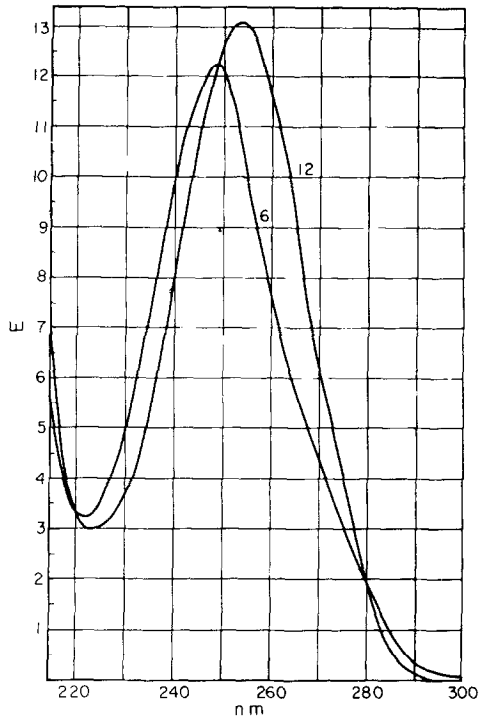


Fig. 4. Ultraviolet spectrum of deoxyinosine at indicated pH values. The deoxyinosine was obtained as a product of demethylamination of N^6-methyldeoxyadenosine with the spleen phosphodiesterase preparation. The data were obtained on a Zeiss PMQII instrument. The spectrum at pH 6 was obtained on an unbuffered H_2O solution that at pH 12 is in 0.01 N NaOH.

was from Worthington Biochemical Corporation; several lots (Code: SPH) have been used, purchased over a period of more than one year. About 0.1 μmole of a nucleoside was digested in a capillary for two hours at 37° with about 30 μl of spleen phosphodiesterase (20 units/ml) in 50 mM Tris HCl buffer, pH 7.4, 20 mM $MgCl_2$. A unit equals change in absorbancy (ΔA_{260}) of 0.2 under the conditions specified in "The Worthington Manual". The digests were spotted on Whatman No. 1 paper and separated electrophoretically in a pyridine/acetic acid buffer, pH 3.5 (5). The ultraviolet spectra and measurements of the kinetics of deamination were obtained on a Zeiss PMQII instrument.

RESULTS AND DISCUSSION. Digestion of deoxyadenosine or N^6-methyldeoxyadenosine with spleen phosphodiesterase under standard conditions gives rise to compounds of zero electrophoretic mobility, indicating a loss of their amino groups and consequent lack of an electric charge at pH 3.5 (Figure 1). Neither the 5'-phosphate (Figure 1) nor the 3'-phosphate (not shown) of N^6-methyldeoxy-

adenosine are substrates for the enzyme. Adenosine (not shown) is deaminated at the same rate as deoxyadenosine. Our previous experience (3) with N^6-[^{3}H] methyldeoxyadenosine (isolated from snake venom phosphodiesterase digests of DNA methylated by the Eco RI methylase) showed that there is also a rapid loss of the [^{3}H]methyl group from the nucleoside when incubated with spleen phosphodiesterase. This indicates that the enzyme can also remove a methylamino group from the purine ring.

The kinetics of disappearance of deoxyadenosine and N^6-methyldeoxyadenosine can be followed spectrophotometrically (Figure 2 and 3, respectively). Although deoxyadenosine is deaminated about 300 times faster than N^6-methyldeoxyadenosine (pH 7.4, 25°), the deamination of both nucleosides is complete and both can be accounted for from the decrease of absorbance at 265 nm or from the increase at 249 nm. Although the molar extinction coefficient for deoxyinosine is not known, it was assumed for the present calculations that the difference in the coefficient at 265 nm (pH 7.4) between deoxyadenosine and deoxyinosine is the same as that between adenosine and inosine (6), i.e. $\Delta\varepsilon_{265}$ = (13,600 - 5,300) = 8,300. Similarly, the difference in the molar extinction coefficient (pH 7.4) between N^6-methyladenosine (7) and inosine, $\Delta\varepsilon_{265}$ = (15,900 - 5,300) = 10,600 was used for the corresponding deoxynucleoside pair to calculate the amount of N^6-methyldeoxyadenosine converted to deoxyinosine. The above assumption is justified because there is practically no difference in the ultraviolet spectrum of a nucleoside and a deoxynucleoside in the acid and neutral pH region (8). From the initial slopes of the curves (Figures 2 and 3) it can be calculated that one unit of the phosphodiesterase preparation deaminates 0.107 μ mole of deoxyadenosine or demethylaminates 3.8×10^{-4} μ moles of N^6-methyldeoxyadenosine per min. at 25°.

The ultraviolet spectrum of the product obtained by spleen phosphodiesterase digestion of N^6-methyldeoxyadenosine is shown in Figure 4, and an identical spectrum has been obtained for the product derived from deoxyadenosine. At pH 6, λ_{max} is at 248.5 nm and λ_{min} at 223 nm. At pH 12, λ_{max} is at 253.5 nm and λ_{min} at 224 nm. The spectrum is practically identical to that of inosine (6). It shows a change in the alkaline pH region, which reflects the dissociation of a proton from the chromophore and which must correspond to the pK_a = 8.8 for inosine (9). Deoxyribonucleosides are only slightly weaker acids (by about 0.1 pH unit) than ribonucleosides and their ultraviolet spectra show practically

the same pH dependence, at least at pH values below 12, where the dissociation of a proton from the ribose moiety can be disregarded (8). It seems safe to conclude, therefore, that the spectrum (Figure 4) represents deoxyinosine.

Two adenosine aminohydrolyses have been previously described, a non-specific enzyme from takadiastase (10, 11) that deaminates adenosine and various adenosine phosphates, and a specific deaminase (E.C. 3.5.4.4.) from various sources (12-15) that is inactive with adenosine phosphates. Different N^6-substituted purine ribonucleosides have been shown to be substrates and inhibitors for adenosine aminohydrolyse (16) but it has not been reported that N^6-methyldeoxyadenosine can also serve as substrate. We do not know whether a single enzymic activity is responsible for both the deaminase and demethylaminase reported here. However, the presence of such an activity in commercial spleen phosphodiesterase preparations must be considered when using this enzyme for studies on nucleotide sequences.

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