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1-Methyladenosine. Dimroth Rearrangement and Reversible Reduction*

James B. Macon and Richard Wolfenden

ABSTRACT: Rearrangement of 1-methyladenosine to 6-methylaminopurine ribonucleoside proceeds at room temperature at a rate proportional to hydroxide ion concentration below pH 8 and above pH 10, with a plateau between. The results are consistent with hydroxide attack on both the protonated and neutral forms of the nucleoside, whose pK_a is 8.25. 1-Methyladenosine is rapidly reduced by sodium borohydride at pH 8.2 to 1-methyl-6-hydroadenosine, pK_a = 11.9. The reduced compound is oxidized by air in alkaline solution to 6-methylaminopurine ribonucleoside, and by nitrous acid at pH 5.4 to 1-methyladenosine. 3-Methylcytidine is also reduced by sodium borohydride, but deamination renders this reaction irreversible.

1-Methyladenine and 3-methylcytidine occur in small quantities in tRNA (Dunn, 1961; Hall, 1967). 1-Methyladenylic acid residues occur in known positions of the nucleotide sequence of yeast tyrosine (Madison *et al.*, 1967) and phenylalanine (RajBhandary *et al.*, 1966) tRNA corresponding to looped-out regions of the secondary structure.

These bases are unusual in being protonated at physiological pH values as discussed below. Modifications of these bases are therefore expected to influence electrostatic interactions in nucleic acids in which they occur, and may also be useful in structural studies. This paper describes two modifications of the free nucleosides, borohydride reduction and Dimroth rearrangement. Both modifications are found to occur at room temperature near neutrality. The product of borohydride reduction of adenosine can be reoxidized selec-

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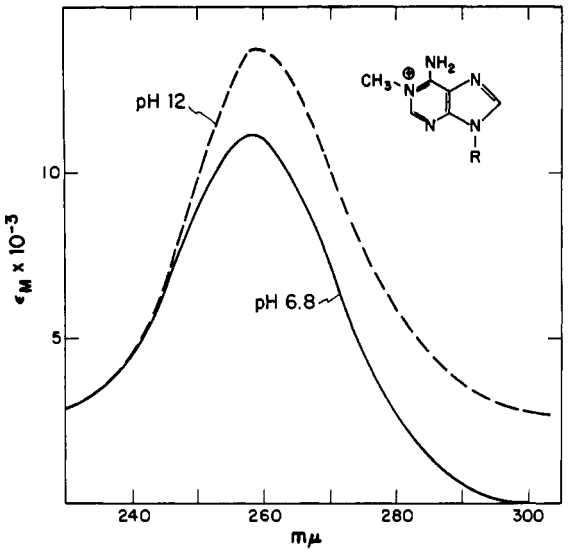


FIGURE 1: Ultraviolet absorption spectra of 1-methyladenosine in 0.1 M potassium phosphate buffers, ionic strength 0.50, 25°.

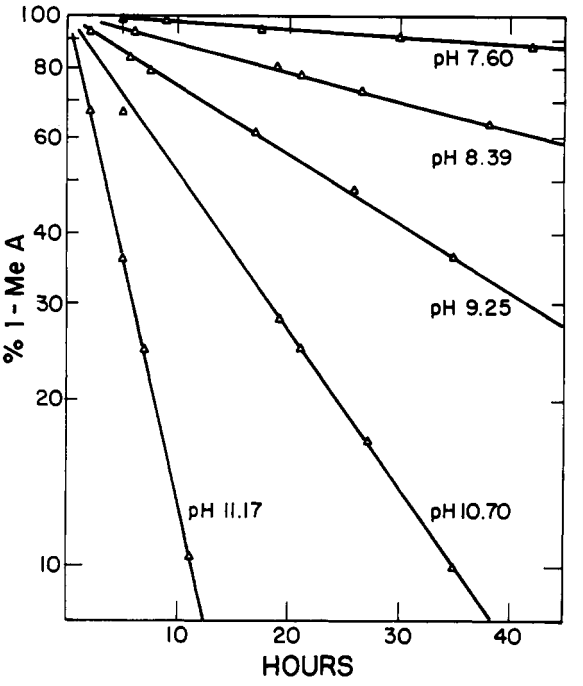


FIGURE 2: Rearrangement of 1-methyladenosine to 6-methylaminopurine ribonucleoside in Tris-HCl and potassium phosphate buffers, 0.1 M, ionic strength 0.50, 25°.

tively, either to 1-methyladenosine or to 6-methylaminopurine ribonucleoside.

Experimental Procedure

1-Methyladenosine was prepared from the hydriodide salt by the method of Jones and Robins (1963). 3-Methylcytidine methosulfate was prepared according to the procedure of Brookes and Lawley (1960). 6-Methylaminopurine ribonucleoside was prepared according to the method of Johnson *et al.* (1958).

Rates of the various reactions were followed in the ultraviolet by measuring the change in absorption of water solutions with a Zeiss PMQ II spectrophotometer equipped with a thermostated cuvet compartment maintained at 25°. Spectra were recorded with a Cary 14 spectrophotometer. Pseudo-first-order rate constants were calculated from the equation $k = 0.693/t_{1/2}$. Ammonia release was measured by the Conway (1958) procedure, conducting the reaction in the outer well of the microdiffusion dish, adding 1 drop of concentrated KOH after completion, and titrating boric acid in the center well after 12 hr. Nuclear magnetic resonance spectra of samples lyophilized and dissolved in D_2O were recorded with a Varian A-60A spectrometer. Chemical shifts are reported in parts per million relative to tetramethylsilane in chloroform. Determinations of pH were made with the glass electrode of a Radiometer 4 pH meter.

Results

Protonation of 1-Methyladenosine. In the presence of alkali 1-methyladenosine undergoes an immediate change in spectrum (Figure 1) which is completely reversible if exposure to alkali is not prolonged beyond 30 min. By comparison of spectra at 300 mμ in 0.5 M Tris-HCl buffers, adjusted to ionic strength 0.50 with KCl, the apparent pK_a value of the conjugate acid of 1-methyladenosine was found to be 8.25 at 25° at ionic strength 0.50. Absorption changes at all wavelengths fitted theoretical titration curves, based on the above pK_a, with reasonable accuracy. Unlike the spectra recorded for the aglycone 1-methyladenine by Brookes and Lawley (1960), no isosbestic point was observed and no higher pK value was evident, presumably due to the absence of a dissociable imidazole proton.

Dimroth Rearrangement of 1-Methyladenosine. Rearrangement of 1-methyladenosine at various pH values was followed by the increase in absorption which occurs upon formation of 6-methylaminopurine ribonucleoside (ϵ_{265} 15.9 × 10^3) (Johnson *et al.*, 1958). Good first-order kinetics were observed at all pH values (Figure 2) and 6-methylaminopurine ribonucleoside was found to be the sole product by ultraviolet absorption spectrum and by paper chromatography. Attempts to trap an aldehyde intermediate with 2,4-dinitrophenylhydrazine (Friedmann and Haugen, 1943) after partial reaction gave negative results. The increase in molar extinction coefficient corresponding to complete conversion varies from 3.5 × 10^3 in alkaline solution to 6.5 × 10^3 at neutrality, but calculation of extinction at infinite time was unnecessary except at the lowest pH values where reaction could not conveniently be followed to completion. At pH 8 and above the reaction was followed in duplicate for at least four half-times with at least six optical density readings.

In Figure 3 the observed pseudo-first-order rate constants, obtained at 25° in 0.1 M Tris-HCl and potassium phosphate buffers, adjusted to ionic strength 0.50, are plotted as a function of pH. No appreciable effect was observed when concentrations of buffers were varied at constant ionic strength.

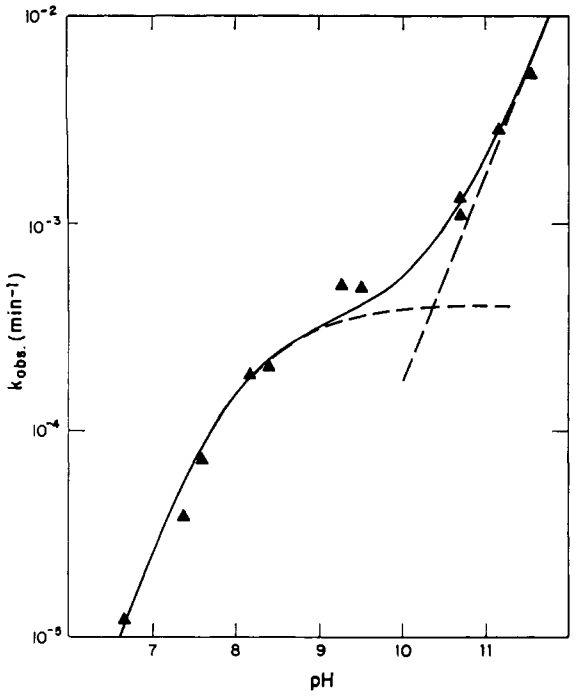


FIGURE 3: Observed rate of rearrangement of 1-methyladenosine to 6-methylaminopurine ribonucleoside, as a function of pH, ionic strength 0.50, 25°. The break in the curve between pH 8 and 10 represents the shift from rate-determining hydroxide attack on the protonated nucleoside to rate-determining attack on the neutral nucleoside.

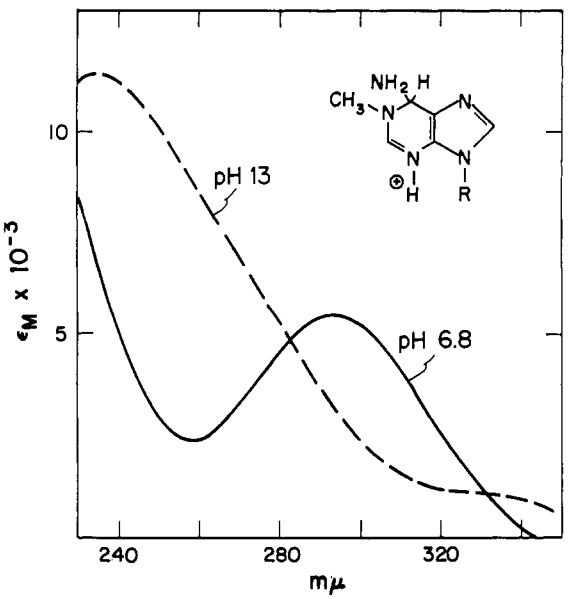


FIGURE 4: Ultraviolet absorption spectra of 1-methyl-6-hydroadenosine in 0.1 M potassium phosphate buffers, ionic strength 0.50, 25°.

This rate profile is consistent with a mechanism involving hydroxide attack on the protonated nucleoside, superseded in importance at high pH by hydroxide attack on the free nucleoside. Assuming nucleoside pK_A = 8.25 at 25° and ionic strength 0.50, a theoretical profile was calculated from the rate law k_{obsd} = 220[OH^-]·[1-MeAH^+] + 1.7[OH^-][1-MeA] min^{-1}. This is shown as the solid line in Figure 3. The dashed lines represent the contributions from the two modes of attack.

Borohydride Reduction of 1-Methyladenosine. A solution of 1-methyladenosine (0.05 M) in 0.2 M Tris-HCl buffer containing 0.1 M sodium borohydride is completely converted in 10 min into a new product, whose properties are described below. Spectra of starting material and product are given, respectively, in Figures 1 and 4. The rate of this reaction was followed under various conditions by removing aliquots, diluting with potassium phosphate buffer (0.1 M, pH 6.5), and observing the appearance of the new product at 292 mμ, its ultraviolet absorption maximum. Analysis by the Conway method showed that deamination did not occur during the reaction. Using 5×10^{-3} M 1-methyladenosine and a large excess of sodium borohydride (0.10 M), apparent first-order kinetics were observed for the reduction in 0.5 M buffers at various pH values, and the half-times for reaction at 25° are shown in Table I.

The reduction product is strongly fluorescent. It yielded 1-methyladenosine (80%) and 6-methylaminopurine ribonucleoside (20%) when subjected to paper chromatography in 1-butanol–water–concentrated ammonia (86:14:5) (Hall, 1967), and the fluorescence disappeared. Later results (see below) showed that reoxidation occurs under alkaline conditions which also lead to Dimroth rearrangement. In more acidic systems the product remained as a fluorescent spot near the origin. For subsequent experiments the borohydride reduction reaction was carried out in ammonia buffer (1 M, pH 9). The solution was adjusted to pH 4 with HCl to destroy the remaining borohydride, was evaporated to dryness, boric acid was removed by repeated evaporations with methanol, and the product was obtained as a mixture with ammonium chloride. Chromatography on Dowex 50 failed to separate this mixture. The presence of NH_4Cl did not interfere with the further analyses described below.

The product moved with a positive charge upon paper electrophoresis at pH 6.8 and 10.1 (0.01 M potassium phosphate buffer). Ultraviolet spectra immediately after

TABLE I: Rates of Reduction by 0.1 M Sodium Borohydride at 25°.

Oxidant	Buffer (0.5 M)	Half-Time (min)
1-Methyladenosine		
0.01 M	Potassium carbonate (pH 10.5)	215
0.01 M	Ammonium chloride (pH 9.5)	35
0.01 M	Tris-HCl (pH 8.65)	4.5
0.01 M	Tris-HCl (pH 8.22)	2
3-Methylcytidine		
0.01 M	Tris-HCl (pH 8.65)	15
0.01 M	Tris-HCl (pH 8.22)	7

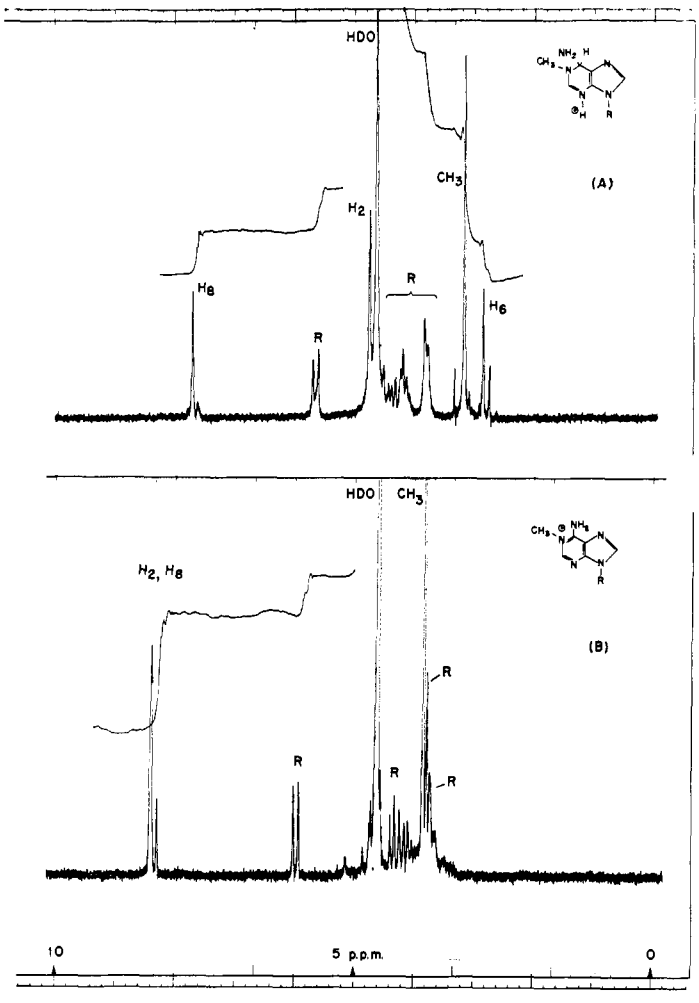


FIGURE 5: Nuclear magnetic resonance spectra in D_2O of (A) 1-methyl-6-hydroadenosine (0.5 M, apparent pH 4.0), and (B) 1-methyladenosine (0.5 M in 0.6 M DCl), at 20°. Chemical shifts in parts per million relative to tetramethylsilane in chloroform. R refers to ribose protons (see text).

dissolving in neutral and alkaline buffers are given in Figure 4. At intermediate pH values the compound undergoes a reversible change in spectrum with isosbestic points at 283 and 331 mμ, indicating a single pK_a, 11.9 at 25° and ionic strength 0.20.

Integrated nuclear magnetic resonance spectra (Figure 5) of 1-methyladenosine at pH 1 (0.5 M solution in 0.6 M DCl in D_2O) showed two protons at 8.40 ppm (H_2 and H_8), a doublet at 6.00 ppm (anomeric proton), a multiplet at 3.6–4.4 ppm (CHOH), and three protons at 3.80 ppm (CH_3). The reduction product (0.5 M solution in D_2O, apparent pH 4.0) showed one proton at 7.70 ppm (H_8), a doublet at 5.66 ppm (anomeric proton), one proton at 4.80 ppm barely resolved from the D_2O peak at 4.60, a multiplet at 3.6–4.4 ppm (CHOH), three protons at 3.14 ppm, and a doublet corresponding to a single proton at 2.80 ppm. The principal change was therefore the disappearance of H_2 from its original position at 8.40 ppm, and the appearance of new peaks at 4.80 and at 2.80 ppm. The nearness of the first of these to the water peak made integration unreliable, but the peak at 2.80 ppm represents a single proton; assignments: 4.80 (H_2) and 2.80 ppm (H_6).

Reoxidation of Hydro-1-methyladenosine. The appearance of 1-methyladenosine and 6-methylaminopurine ribonucleoside during paper chromatography in alkali indicated that reoxidation had occurred. In 10% ammonia or in 0.1 M potassium carbonate buffer (pH 11.0) the compound is quantitatively converted into 6-methylaminopurine ribonucleoside with a half-time of approximately 40 min at 25°. Exposure to ultraviolet radiation does not appreciably affect the rate of this reaction. At lower pH values the reaction proceeds more slowly, but yields increasing proportions of 1-methyladenosine. Quantitative oxidation to 1-methyladenosine occurs rapidly upon exposure of the compound to sodium nitrite (0.03 M) in sodium acetate buffer (1 M, pH 5.4) at room temperature, with a half-time of approximately 3 min. No deamination occurs under these conditions. The quantitative nature of borohydride reduction and reoxidation provided the basis for the molar extinction coefficients recorded for the reduction product in Figure 4.

Borohydride Reduction of 3-Methylcytidine. Under the same conditions as those described for 1-methyladenosine, 3-methylcytidine is rapidly reduced by sodium borohydride (Table I). Analysis by the Conway method showed that 1 mole of ammonia was released during the

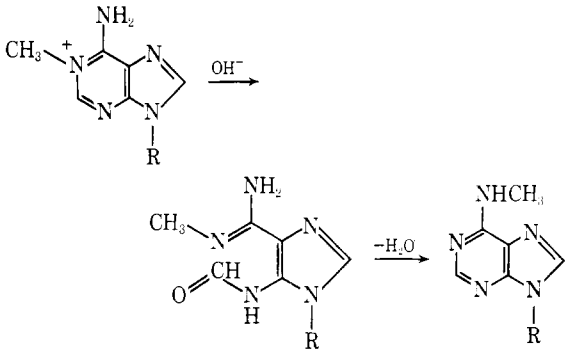
course of the reaction. The other product shows only end absorption in the ultraviolet, with a molar extinction coefficient of 4.5×10^3 at 230 mμ (pH 7) compared with 11.0×10^3 for 3-methylcytidine. As expected from the loss of ammonia, no reoxidation to cytidine derivatives occurred when the reduction product was exposed to alkali or to nitrous acid under the conditions described above.

Discussion

The pK_a value of the conjugate acid of 1-methyladenosine, 8.25, may be compared with values recorded (Brookes and Lawley, 1960) for the protonated aglycones 1-methyladenine (7.2) and 3-methylcytidine (7.4) (Brookes and Lawley, 1962). 1-Methyladenylic acid residues, which have been found in the looped-out regions of the sequence of yeast tyrosine (Madison *et al.*, 1967) and phenylalanine (RajBhandary *et al.*, 1966) tRNA, are thus expected to possess a positive charge on the ring at neutral pH. 3-Methylcytidylic and 6-aminoacyladenylic acid residues, which are known to be present in yeast tRNA (Hall, 1967), presumably share this property, although their positions in pure species of tRNA remain to be established.

Brookes and Lawley (1960) showed that 1-methyladenine and 1-methyladenylic acid undergo Dimroth rearrangement to the corresponding 6-methyladenine derivative in alkaline solution, and Jones and Robins (1963) demonstrated a similar rearrangement of 1-methyladenosine. These reactions were performed in strong alkali at elevated temperatures. The present results show that rearrangement takes place at room temperature in neutral solution. The rate of this reaction, which is presumed to occur through ring opening by analogy with rearrangements of N-methylated pyrimidines (Brown, 1961), tends toward a plateau above the pK_a of the nucleoside, increasing again at high pH values (Figure 1). The reaction may proceed through water attack on the neutral species, or through the kinetically indistinguishable attack by hydroxide ion on the protonated nucleoside. We favor the latter alternative, since water acting as a nucleophile would be expected to attack the protonated species more rapidly than the neutral species, and no such reaction is in fact observed. Attack by hydroxide ion on the neutral species becomes important at high pH, as postulated for a similar rearrangement by Taylor and Loeffler (1960). The ratio of the rate constants for hydroxide attack on the protonated and neutral species, 130, may be compared with ratios of 170 and 140 observed for hydroxide attack on the protonated and neutral species of leucyl-tRNA and leucine ethyl ester (Wolfenden, 1963).

The ring-opening step appears to be rate determining, since no intermediate was detected by spectrophotometry, by trapping with 2,4-dinitrophenylhydrazine, or by analysis of the pH–rate dependence of the reaction. If ring closure were rate determining, the rate of the reaction would be expected to show a simple dependence upon the state of ionization of the intermediate, whose pK_a value should resemble those of other amidines, which are near 12 (Albert *et al.*, 1948).



Leonard *et al.* (1966) found that the rearrangement of 1-(γ,γ-dimethylallyl)adenine at 100° at pH 7.5 proceeded with a half-time of the order of 1 hr, and Grimm and Leonard (1967) have reported half-times of approximately 40 and 60 min for the ribonucleoside and 5′-ribonucleotide, respectively, in concentrated ammonia at 60°. From these results it appears likely that the dimethylallyl derivative rearranges somewhat more slowly than does the methyl derivative. The present results confirm the need for extreme caution in work-up procedures for polynucleotide sequence analysis. The successful assignment of sequence positions for 1-methyladenylic acid in yeast tyrosine and phenylalanine tRNA indicate that the necessary precautions were taken in those analyses. Deliberate induction of the Dimroth rearrangement at neutral pH should be useful in investigations of the function of 1-methyladenylic acid residues in tRNA since it results in a nucleotide residue with no net charge and altered hydrogen-bonding capabilities.

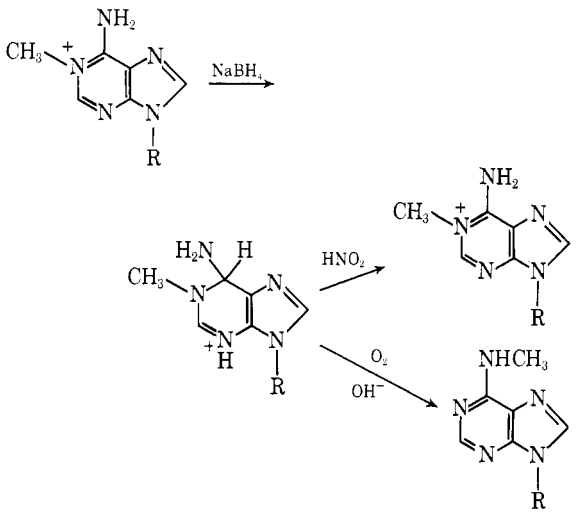
Another potentially useful characteristic of 1-methyladenosine is its susceptibility to reduction by borohydride. The product reverts to the starting compound upon oxidation with nitrous acid. In alkaline solution it is oxidized by air with rearrangement to yield 6-methyladenosine.

Hydride attack might be expected at either the 2 or the 6 position, with the 6 position possibly preferred on electrostatic grounds (Albert, 1959; Pullman, 1959; Barlin and Chapman, 1965). In either case the product would possess amidinium resonance in its protonated form, explaining the high pK_a value observed, approximately 12. Either position of attack would lead to the observed upfield shift of the C-2 proton, but attack at C-2 would be expected to double its intensity. Instead, the product possesses two new peaks, 3.6 and 5.6 ppm upfield, indicating that C-6 is the actual site of attack. The ultraviolet absorption maximum of the product (292 mμ) is the same as that of 1,6-dihydropurine obtained by electrochemical reduction of purine (Smith and Elving, 1962).

The ultraviolet absorption of the product (292 mμ) is the same as that of the product obtained by electrochemical reduction of purine (Smith and Elving, 1962). It thus tends to confirm their conclusion that electrochemical reduction of purine occurs across the 1,6 double bond, based on the appearance of diazotizable amino groups at various stages of reduction. Skulachev (1963) has postulated direct involvement of adenine nucleotides in the generation of high-energy phosphate

bonds in oxidative phosphorylation, and suggests 1,6-dihydroadenine derivatives as intermediates. This suggestion is based on comparison of difference spectra of reduction of mitochondria with the difference spectrum of reduction of purine obtained by Smith and Elving.

Smith and Elving (1962) found that electrochemical reduction of adenine in acid, proceeding through a hypothetical intermediate similar to the present product, was accompanied by 1,6 elimination of ammonia so that 1,6-dihydroadenine could not be isolated. In the case of 1-methyl-6-hydroadenosine this elimination is blocked.



A close analog of the present reaction is the recently reported borohydride reduction of 1,3,7,9-tetramethyl-8,-9-dihydroxanthine, which is also reversed by oxidizing agents (El'tsov and Muravich-Alexander, 1968).

Reduction of 3-methylcytidine with borohydride led to elimination of ammonia and is thus irreversible. The structure of the other product, which possesses only end absorption in the ultraviolet region, was not determined. Other nucleic acid bases already shown to undergo irreversible reduction by borohydride are 4,5-dihydrouracil, 6-acetylcytosine, and 6-thiouracil (Cerutti and Miller, 1967).

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