Mechanism of Hydroxylamine Mutagenesis: Tautomeric Shifts and Proton Exchange between the Promutagen N^6-Methoxyadenosine and Cytidine†

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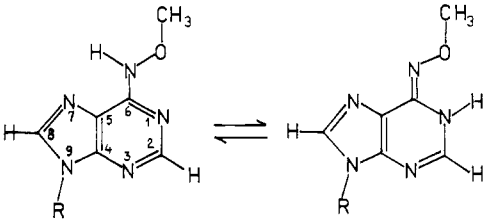
ABSTRACT: Whereas the amino, but not imino, tautomer of the promutagen N^6-methoxyadenosine (OMe^6A) forms planar associates (base pairs) with the potentially complementary uridine [Stolarski, R., Kierdaszuk, B., Hagberg, C.-E., & Shugar, D. (1984) *Biochemistry 23*, 2906–2913], it has now been found, with the aid of ^1H NMR spectroscopic techniques, that only the imino tautomer of OMe^6A base pairs with the potentially complementary cytidine. The association constant for such heteroassociates is more than an order of magnitude higher than that for autoassociates of OMe^6A. The formation of heteroassociates is accompanied by a marked shift in tautomeric equilibrium of OMe^6A, with an increase in the population of the amino form from 18% to as high as 44% and a corresponding decrease in the population of the imino species. Furthermore, the presence of cytidine in a solution of OMe^6A appreciably enhances the rate of tautomeric exchange between the two tautomeric forms. Formation of planar heteroassociates between cytidine and the imino form of OMe^6A is also accompanied by proton exchange between the cytidine NH_2 and the N^6-H of the amino form of OMe^6A. The rate constants for this exchange and for tautomeric exchange, determined by the saturation transfer technique, have been measured at various concentrations and temperatures. A model is advanced for proton exchange that takes into account the interdependence of tautomeric exchange and proton exchange, as well as the role of auto- and heteroassociates. The relevance of these results to the molecular basis of hydroxylamine and methoxyamine mutagenesis and to the phenomenon of proton exchange in other systems is briefly discussed.

The N^6-hydroxy and N^6-methoxy derivatives of adenosine, the products of reaction of adenosine with the mutagens hydroxylamine (NH_2OH) and methoxyamine (NH_2OCH_3) [see Budowsky (1976), Marfey and Robinson (1981), and Shugar and Kierdaszuk (1985)], are promutagens in various procaryotic (Freese, 1968; Budowsky et al., 1975) and eucaryotic systems, e.g., in yeast (Pavlov & Khromov-Borisov, 1984), in *Escherichia coli* (Salganik et al., 1973), in *Neurospora crassa* (Brockman et al., 1979; Overton et al., 1981; Mc Cartney et al., 1985), and in Syrian hamster embryo fibroblasts (Barrett, 1981). Apart from their properties as promutagens, the N^6-hydroxy derivatives of a number of adenosine analogues have been found to exhibit significant in vitro antitumor and antiviral activities (Giner-Sorolla et al., 1981).

The promutagenic properties of these analogues are further testified to by the fact that, in in vitro RNA and DNA polymerase systems (Singer & Spengler, 1982; Thabit Abdul-Masih & Bessman, 1986), each behaves like both A and G; i.e., they exhibit dual functionality. In this respect they resemble the promutagenic N^4-hydroxy and N^4-methoxy derivatives of cytidine (Budowsky et al., 1971; Flavell et al., 1974), which exist in aqueous medium as an equilibrium mixture of two tautomeric species (Brown et al., 1968; Sverdlov et al., 1971; Morozov et al., 1982) and in the imino form in aprotic solvents (Brown et al., 1968; Kierdaszuk & Shugar, 1983; Kierdaszuk et al., 1983a), in the gas phase (Kulińska et al., 1980), and in the solid state (Shugar et al., 1976; Birnbaum et al., 1979).

Chart I: Amino–Imino Tautomers of N^6-Methoxyadenosine[a]



[a] R = ribose or 2′,3′,5′-tri-*O*-methylribose to increase solubility in nonaqueous media. Shown is the syn rotamer, with the N^6-OCH_3 group syn to the ring N(1).

It was previously shown that the dual functional properties of N^6-methoxyadenosine (OMe^6A)[1] are equally reflected by its existence, in aqueous or low-polar and aprotic media ($CHCl_3$, CCl_4), as an equilibrium mixture of two tautomers, amino and imino (Chart I), the tautomeric equilibrium being strongly solvent dependent (Kierdaszuk et al., 1983b, 1984; Stolarski et al., 1984). Furthermore, the amino, but not imino, form of OMe^6A formed planar hydrogen-bonded associates (base pairs) with the potentially complementary uridine (Chart II), and in fact, addition of uridine to a solution of OMe^6A led to an increase in the amino population of the latter, with a corresponding decrease in the population of the imino species (Stolarski et al., 1984). It was additionally noted that OMe^6A also formed a base pair with the potentially complementary cytidine. We have now extended this observation to show that

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[1] Abbreviations: OMe^6A, N^6-methoxyadenosine; C, cytidine.

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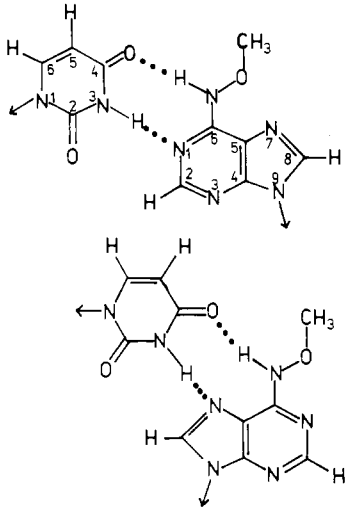
Chart II: Proposed Structures of Hydrogen-Bonded Planar Heteroassociates of Uridine and the Amino Tautomer of OMe^{6}A with the N^6-OMe Group of the Latter in the Anti (Upper) or Syn (Lower) Conformation Relative to the Adenine N(1)[a]



[a] Arrows indicate glycosidic linkages. Note that base pairing involves the uracil C(4)=O, but rotation of the OMe^{6}A molecule by 180° about an axis through the uracil C(6) and N(3) will lead to similar heteroassociates via the uracil C(2)=O.

the imino, but not amino, form of OMe^{6}A forms planar associates with C, again accompanied by a shift in tautomeric equilibrium and, additionally, an exchange of N–H-type protons between the two bases. The relevance of these phenomena to the mechanism of A → G transitions via N^6-methoxyadenosine in methoxyamine mutagenesis prompted us to undertake a detailed study with the aid of NMR spectroscopy.

Materials and Methods

The 2′,3′,5′-tri-*O*-methyl derivative of N^6-methoxyadenosine and the 2′,3′,5′-tri-*O*-ethyl derivative of cytidine were synthesized as described elsewhere (Kierdaszuk et al., 1984; Kuśmierek et al., 1973).

The *sole* purpose of alkylation of the sugar hydroxyls of the two nucleosides was to enhance their solubility in low-polar solvents such as chloroform, widely employed in studies on planar association, i.e., base pairing via hydrogen bonding (Iwahashi & Kyogoku, 1977, 1980).

^{1}H spectra for compounds in C^2HCl_3 (Merck, Darmstadt, GFR, 99.5% mol ^{2}H, dried over A4 molecular sieves) were recorded on Bruker 270 AM and Jeol 200 XL spectrometers, relative to internal Me_4Si to an accuracy of ±0.005 ppm.

Saturation transfer measurements were performed by recording 16 scans at low-power on-resonance selective irradiation during the entire period except that for signal acquisition, followed by 16 scans at off-resonance irradiation as in measurements of the Overhauser effect [e.g., see Sanderson et al. (1983)].

Measurements of relaxation times T_1 for protons undergoing exchange, by selective irradiation of the signal of the proton with which this exchange occurs (Alger & Schulman, 1984), were performed by the method of inversion recovery (Farrer & Becker, 1971). Selective irradiation was maintained during the entire period, except that for signal acquisition.

Selective irradiation of the peak of the proton of the form Y leads to a decrease in intensity of the peak of the corresponding proton of the form X, viz., $f_X(Y) = [I_X - I_X(Y)]/I_X$ (Perrin & Johnston, 1979) during the exchange X ⇌ Y, slow on the NMR time scale. Profiting additionally from the measurement of the relaxation time of this proton in the form X by selective irradiation of the appropriate proton of the form Y, $T_{1X}(Y)$, one may determine the rate constants for the transition X → Y (Perrin & Johnston, 1979; Agler & Schulman, 1984):

$$k_{XY} = f_X(Y)T_{1X}(Y)^{-1} \quad (1)$$

Errors of the estimated rate constants were evaluated, from the errors of signal integrations and errors in measurements of T_1, at about 10%.

The ^{1}H spectra of OMe^{6}A and C and their equimolar mixtures in chloroform at various concentrations and temperatures were recorded on the Jeol XL 200. From the concentration dependence of the chemical shifts of the protons of the amino group of C and of the N^6-H of the amino and the N(1)-H of the imino species of OMe^{6}A (Chart I), the auto- and heteroassociation constants were calculated according to the procedure of Iwahashi and Kyogoku (1977).

Apparent autoassociation constants K_a are defined as

$$X + X \rightleftharpoons XX$$

$$K_a = \frac{C_{XX}}{C_X^2} = \frac{(\delta_m - \delta_X)(\delta_{XX} - \delta_X)}{2C_X^0(\delta_{XX} - \delta_m)^2} \quad (2)$$

where X = C, amino OMe^{6}A, or imino OMe^{6}A. Apparent heteroassociation constants K_a' are defined as

$$X + Y \rightleftharpoons XY$$

$$K_a' = \frac{C_{XY}}{C_X C_Y} = \frac{(\delta_m - \delta_X)(\delta_{XX} - \delta_X)}{C_X^0(\delta_{XY} - \delta_m)} \quad (3)$$

where the heteroassociates XY = C:OMe^{6}A, amino OMe^{6}A:C, or imino OMe^{6}A:C. δ_X, δ_{XX}, and δ_{XY} are the limiting chemical shifts for X, XX, and XY; δ_m is the observed chemical shift. C_X^0 is the overall concentration of X = C, amino OMe^{6}A, or imino OMe^{6}A (the concentrations of the latter two corrected from the tautomeric equilibrium under the experimental conditions). C_X, C_{XX}, C_Y, and C_{XY} are the concentrations of X, XX, Y, and XY.

The limiting chemical shifts δ_X, δ_{XX}, and δ_{XY} and the attendant apparent association constants were the parameters determined by fitting eq 2 and 3 to the experimental points (Figure 1) with the aid of a computer program (Selkers, 1985). Because of the disappearance of the signals of the amino group of C and of the N^6-H of amino OMe^{6}A, at temperatures above −30 °C, due to exchange effects, auto- and heteroassociation constants were determined at −40 °C from the concentration dependences of both signals of the amino group of C and of the signals of N^6-H and N(1)-H of the amino and imino forms, respectively, of OMe^{6}A.

It is unfortunate that the foregoing procedure is not applicable at temperature above −30 °C. The use of infrared spectroscopy, succesfully applied elsewhere (Kierdaszuk et al., 1984), would in this system be too complex because of the expected presence of eight overlapping N–H stretching bands.

Results

Auto- and Heteroassociation Constants of OMe^{6}A and C. Previous qualitative data on planar complexes of the tautomeric forms of N^6-methoxyadenosine with potentially complementary bases (Stolarski et al., 1984) suggested that planar associates occurred between C and OMe^{6}A and led to the present detailed studies.

The chemical shifts of the N–H-type protons in C, OMe^{6}A, and equimolar mixtures of the two in chloroform exhibit a sigmoidal dependence on the concentration of the individual components (Figure 1). This reflects formation of hydro-

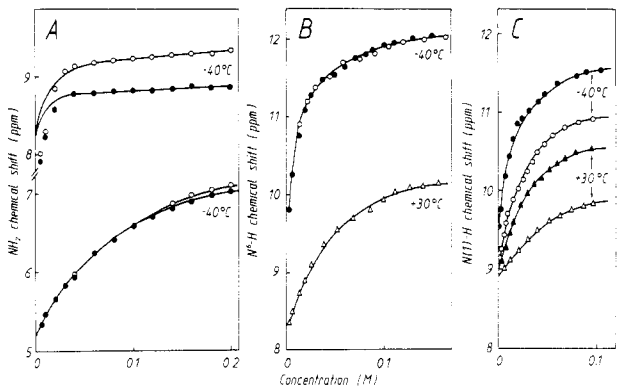
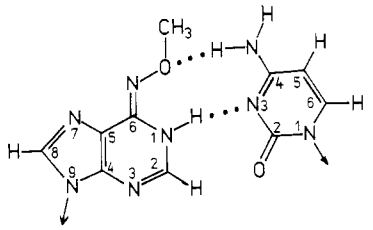


FIGURE 1: Concentration dependence, in chloroform solution, of the ^1H chemical shifts of (A) the downfield and upfield protons of the NH_2 of C(○) and its 1:1 mixture with OMe^6A (●) at −40 °C, (B) the N^6-H of amino OMe^6A at −40 °C (○) and at 30 °C (△) and its 1:1 mixture with C at −40 °C (●), and (C) the N(1)-H of imino OMe^6A at −40 °C (○) and at 30 °C (△) and its 1:1 mixture with C at −40 °C (●) and at 30 °C (▲). Note: The continuous lines denote the computer-fitted theoretical curves. In (A), concentrations refer to each of the components. In (B) and (C), concentrations refer to the amino and imino forms of OMe^6A.

Chart III: Proposed Structure of the Hydrogen-Bonded Planar Heteroassociate of the Imino Tautomer of OMe^6A with Cytidine[a]



[a] Note that the N^6-OCH_3 group is syn relative to the adenine N(1). Arrows indicate the glycosidic bonds.

gen-bonded planar auto- and heteroassociates, in which exchangeable N–H groups are proton donors. An increase in concentration, and/or a decrease in temperature, leads to an increase in the extent of auto- and/or heteroassociation, reflected in downfield shifts of the signals of the N–H groups involved in hydrogen bonding. This confirms our previous prediction regarding the structure of the heteroassociate between C and imino OMe^6A (Chart III), in which the N^6–OCH_3 group in syn with respect to the adenine ring N(1), which is the dominant rotamer in solution (Stolarski et al., 1984) and the form observed in the solid state (Birnbaum et al., 1984).

From the concentration dependence of the chemical shifts of the N–H-type protons, determinations were made of the autoassociation constants (eq 2) of C and the individual tautomeric species of OMe^6A and of the heteroassociation constants (eq 3) of C with each of the tautomeric forms of OMe^6A (Table I). These constants are, of course, only apparent values, since the calculations took no account of different types of complexes (Stolarski et al., 1984). Calculations of heteroassociation constants took no account of the effects of autoassociation of the individual components, and in the case of autoassociation constants of the tautomeric species and their heteroassociates with C, heteroassociation between tautomeric species was ignored. Consequently, the calculated apparent association constants reflect the overall involvement of a given proton in hydrogen bonding, without specifying the type of planar complex formed and on the assumption that 1:1 stoichiometry prevailed.

Comparisons of the auto- and heteroassociation constants (Table I) demonstrate, independently of temperature, pref-

Table I: Apparent Association Constants for Autoassociates of C and OMe^6A and Heteroassociates between Them Derived from the Concentration Dependence of the Chemical Shifts of Indicated Protons

		self-association constants (M^{-1})		heteroassociation constants (M^{-1}) of OMe^6A with C	
compd	proton	−40 °C	30 °C	−40 °C	30 °C
C	NH_2 d[a]	77	*b*		
C	NH_2 u[a]	3	*b*		
amino OMe^6A	N^6-H	79	9	84	*b*
imino OMe^6A	N(1)-H	11	3	72	16

[a] Symbols d and u refer to the downfield and upfield locations of the NH_2 protons. [b] Disappearance of the signals of the NH_2 of C and of the N^6-H of amino OMe^6A in the 1:1 complex of the latter with C (see Materials and Methods) precluded determination of association constants at temperatures above −30 °C.

erential heteroassociation of C with the imino form of OMe^6A (association constant 72 M^{-1} at −40 °C and 16 M^{-1} at 30 °C) relative to autoassociation of this tautomeric species (11 M^{-1} at −40 °C and 3 M^{-1} at 30 °C). This is fully analogous to the frequently reported preferential formation of the heteroassociates (base pairs) A:U and G:C relative to formation of autoassociates by each of the individual components [Petersen and Led (1981) and references cited therein].

Analyses were also performed of the concentration dependence of the chemical shifts of the two NH_2 protons of C, viz., the downfield (d), which participates directly in hydrogen bonding in 1:1 complexes, and the upfield (u) proton, in equimolar mixtures of C and OMe^6A (Figure 1). The NH_2 d proton was found to exhibit anomalous behavior, relative to the other protons involved in hydrogen bonding in that, following addition of OMe^6A, it underwent shielding in place of deshielding. Furthermore, fitting of the theoretical curve (eq 3) to the experimental values of the chemical shifts of NH_2 d was unsatisfactory, particularly in the concentration range 0.005–0.02 M, precluding calculations of reliable values of heteroassociation constants.

The foregoing two effects are most likely due to use of a simplified model of interaction between OMe^6A and C, on the basis of formation of only 1:1 associates and neglect of autoassociates of C during analysis of heteroassociation processes. Involvement of higher order associates than 1:1 is probably appreciable at higher concentrations. It is true that the overall ratio of C:OMe^6A = 1, but the real ratio of C:imino OMe^6A exceeds 2.5, depending as it does on the tautomeric equilibrium of OMe^6A. It is not feasible to approximate conditions with equimolar equivalents of C and imino OMe^6A without an appreciable increase in the concentration of the latter, which would lead to a marked increase in involvement of its autoassociates. Hence, the concentration range for fitting the theoretical curve to the chemical shifts of the cytidine protons is much higher than that for imino OMe^6A and falls in the range where participation of higher order heteroassociates is marked.

The high autoassociation constant of C is comparable to the heteroassociation constant of C with imino OMe^6A (Table I). Consequently, fitting of the theoretical curve for heteroassociation from eq 3 is, in this instance, an oversimplification since effects of autoassociation can not be ignored.

Effect of Planar Association (Base Pairing) on Tautomeric Equilibrium of N^6-Methoxyadenosine. It was previously shown (Kierdaszuk et al., 1983; Stolarski et al., 1984) that uridine base pairs uniquely with the amino form of OMe^6A

Table II: Percentage Populations of Amino (P_a) and Imino (P_i) Tautomers of N^6-Methoxyadenosine in Chloroform Solution at Various Concentrations and Temperatures and Percentage Populations following Addition of an Equimolar Amount of the Potentially Complementary Cytidine[a]

concn (M)	−40 °C				−30 °C			
	free		mixt		free		mixt	
	P_a	P_i	P_a	P_i	P_a	P_i	P_a	P_i
0.04	76	24	62	38	67	33	63	37
0.10	75	25	60	40	68	32	59	41
0.20	82	18	56	44	70	30	*b*	*b*

[a] Population values accurate to 2%. [b] Population values indeterminate because of overlapping of signals of the two tautomeric species under these conditions.

and that addition of an equimolar quantity of uridine to a chloroform solution of OMe^6A led to enhancement of base pairing via a marked shift in the tautomeric equilibrium of the latter, viz., an up to 10% increase in population of the amino form and a concomitant decrease in population of the imino form.

We have now confirmed that the imino, but not the amino, form of OMe^6A forms planar associates with cytidine. Furthermore, in the presence of an equimolar amount of cytidine, the tautomeric equilibrium of OMe^6A also undergoes an appreciable shift (Table II). Under conditions favoring association, i.e., elevated concentration and/or low temperature, the population of the imino form increases as much as 26%, with a corresponding decrease in the population of the amino species.

Proton Exchange between Amino and Imino OMe^6A. The amino (A) and imino (I) tautomers of OMe^6A undergo slow exchange (A $\rightleftharpoons$ I), on the NMR time scale, with rate constants k_{AI} (A $\rightarrow$ I) and k_{IA} (I $\rightarrow$ A). This effect may be followed directly through saturation transfer between protons of the aglycon (and between protons of the sugar ring) of both forms, which interconvert during the transition from one form to the other (see Materials and Methods).

Determinations of k_{IA} and k_{AI} (eq 1) were based on measurements of the changes in integral intensity and relaxation times of the H(8) proton of the forms A and I [f_A(I) and f_I(A) of the order of 0.2–0.7, and T_1 of the order of 0.5–1 s, depending on the proton and solute concentration]. The values of k_{AI} and k_{IA} for concentrations of 0.04 and 0.2 M at 30 °C are listed in Table III. It will be seen that k_{IA}/k_{AI} is close to the value of the tautomeric equilibrium constant, determined from measurements of the population ratio p_A/p_I, at a concentration of 0.04 M. There is some divergence at 0.2 M (k_{IA}/k_{AI} = 1.9 and p_A/p_I = 2.3 at 30 °C), but this is still within the limits of error of the value of k. The 5-fold increase in concentration from 0.04 to 0.2 M leads to a 4.5-fold increase in the rate constant for exchange, pointing to a concentration dependence of tautomeric exchange (see Discussion, below).

Decrease of the temperature to −40 °C slows down the rate of exchange between tautomers, to the point where no saturation transfer effect is observed. On the assumption of the absence of marked changes in the free enthalpy, $\Delta G^{\ddagger}$, of the barrier between tautomers as a function of temperature, the values of k_{AI} and k_{IA} at −40 °C may be approximated to about 10^{-5}–10^{-4} s^{-1}.

Exchange Processes in Equimolar Mixture of OMe^6A and C. In equimolar mixtures of the two compounds, several exchange processes occur, as is evident from the spectrum at 30 °C (Figure 2). Absence of the NH_2 signals of cytidine is related to hindred rotation about C(4)–N (Becker et al., 1965; Shoup et al., 1966, 1971), and the similar disappearance of the N^6-H of amino $OMe^6A(Me)_3$ is due to involvement of this proton in exchange processes at a rate intermediate on the NMR time scale. In addition, the ^1H and ^{13}C signals

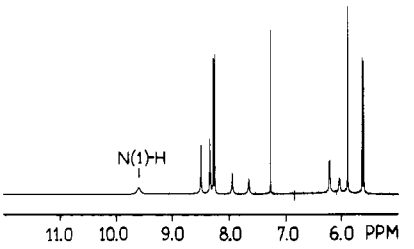


FIGURE 2: ^1H spectrum of an equimolar mixture of OMe^6A and C, each at a concentration of 0.04 M, at 30 °C. Chemical shifts are vs. internal Me_4Si.

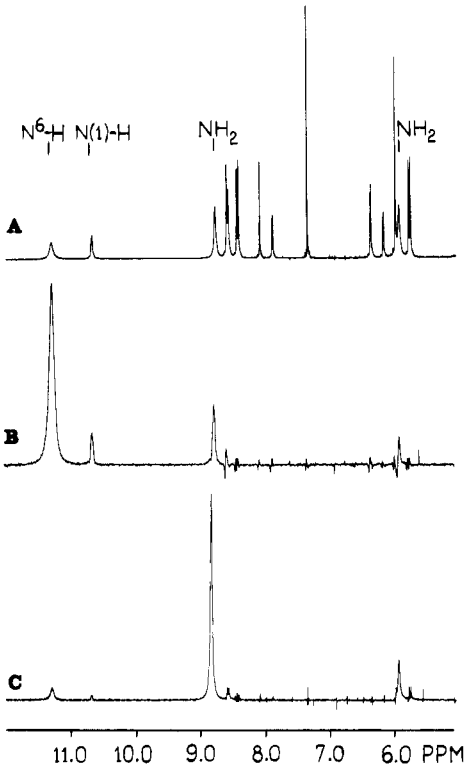


FIGURE 3: ^1H spectrum of (A) an equimolar mixture of OMe^6A and C, each at 0.04 M, at −40 °C, and the difference spectra demonstrating the effects of saturation transfer on selective irradiation of (B) the N^6-H of amino OMe^6A and (C) the downfield signal of the NH_2 of C.

(Stolarski et al., 1984) are broadened. As the temperature is gradually lowered to −40 °C, the NH_2 and N^6-H signals become visible, under conditions of slow exchange on the NMR time scale (Figure 3). Selective irradiation of the appropriate protons at −40 °C reduces the intensities of the signals of those protons that undergo exchange with them. Strong saturation transfer effects are observed between the protons of the amino and imino forms of $OMe^6A(Me)_3$ [f_A(I) and f_I(A) ~ 0.1], as against $OMe^6A(Me)_3$ alone, for which at this temperature there is no observed saturation transfer (see above). Very marked mutual saturation transfer effects occur for the N^6-H

Table III: First-Order Rate Constants, k_{XY}, for Exchange Process of the Type X → Y Derived from Measurements of Saturation Transfer and Relaxation Times T_1: X and Y = A (Amino OMe^{6}A), I (Imino OMe^{6}A), and C

	analogue and conditions of measurement				
k_{XY} (s^{-1})	OMe^{6}A (0.04 M, 30 °C)	OMe^{6}A (0.2 M, 30 °C)	OMe^{6}A + C (0.04 M each, 30 °C)	OMe^{6}A + C (0.04 M each, −40 °C)	OMe^{6}A + C (0.1 M each, −40 °C)
k_{AI}	0.20	0.90	4.0[a]	0.17	0.21
k_{IA}	0.38	1.70	4.5[a]	0.29	0.28
k_{AC}			[b]	0.6[c]	0.5[c]
k_{CA}			[b]	0.3[c]	0.3[c]
k_{CC}			[b]	1.30	1.70

[a] Because of signal broadening, these values are of lower accuracy. [b] Appropriate signals absent, due to exchange on NMR intermediate time scale. [c] Approximate estimates, due to lack of T_1 values for the amino N^6-H and the N(3)-H of C on selective irradiation of all proton signals involved in exchange, except for the proton for which measurement was made.

proton of the amino form and one of the cytidine NH_2 protons, which is located downfield and participates in hydrogen bonding in heteroassociates [f_A(C) and f_C(A) about 0.1–0.15]. Weak saturation transfer effects (f about 0.02) are noted between the N^6-H and the upfield cytidine NH_2 proton and between the N(1)-H of imino OMe^{6}A and the downfield proton of the C NH_2 (Figure 3).

The following two points are of some interest. Proton exchange occurs between the cytidine NH_2 and the N^6-H of amino OMe^{6}A, i.e., the form that does not enter into heteroassociates with C. The presence of the latter strongly accelerates the exchange process A ⇌ I, so that saturation transfer is observed at −40 °C. Acceleration of this exchange, albeit to a lesser extent, is also visible at 30 °C, leading to broadening of the ^{1}H and ^{13}C signals (see Table III).

Determination of rate constants for exchange between N states, when $N > 2$, is rather complex and requires formulation of a defined mechanism. Analysis of the equations for the z components of the spin magnetization under conditions of proton exchange between more than two states (Perrin & Johnston, 1979) indicates the possibility of saturation transfer effects between two states not directly involved in exchange. In such instances, saturation transfer occurs via a third state, with which the preceding two exchange protons directly. Weak saturation transfer effects (see above) between the proton of amino OMe^{6}A and the upfield NH_2 proton of C and between the proton of imino OMe^{6}A and the downfield proton of the NH_2 of C are probably due to such an indirect magnetization transfer. On the basis of this assumption, one may readily determine the rate constants for rotation of the amino group, k_{CC}, and for tautomeric exchange, k_{AI} and k_{IA} (Table III). At a concentration of 0.04 M, $k_{CC} \sim 1.3$ s^{-1}, hence fairly close to the value of 1.1 s^{-1} determined for C alone in the absence of OMe^{6}A. The values of k_{AI} and k_{IA} are identical at concentrations of 0.04 and 0.1 M (Table III) and 4 orders of magnitude higher than those evaluated in the absence of C at −40 °C. In the presence of C at 30 °C, there is a 10-fold increase in the rate of exchange A ⇌ I.

The rate constants for intermolecular proton exchange between the N^6-H of amino OMe^{6}A and the NH_2 of C may be represented as

$$\text{H–N}^6\text{OMeA} + \text{HNH–C} \underset{k'_{CA}}{\overset{k'_{AC}}{\rightleftharpoons}} \text{H–N}^6\text{OMeA} + \text{HNH–C}$$

and are clearly second-order constants. However, these could not be determined *directly*, due to the lack of values for the relaxation times T_1 for the N^6–H and the downfield NH_2 d proton on selective irradiation of the two remaining exchangeable protons [i.e., the N(1)-H and the downfield NH_2 proton and the N^6-H and the upfield proton, respectively], since the equipment at our disposal did not permit *simultaneous* irradiation of two exchangeable protons. This difficulty was circumvented by irradiation in turn of each of the exchangeable protons. Fortunately, the two values of T_1 thus obtained (0.37 and 0.39 s) turned out to be sufficiently close to each other so that, in accord with Perrin and Johnston (1979), the mean value of T_1, 0.38 s, is credible. The resulting apparent first-order rate constants thus determined for exchange, k_{AC} (exchange of proton A → C from N^6–H to NH_2) and k_{CA} (C → A), were reasonably close to each other when measured at concentrations of 0.04 and 0.1 M (Table III).

Discussion

Formation of heteroassociates between C and imino OMe^{6}A leads to proton exchange between the cytidine NH_2 proton that is syn relative to the ring N(3) and the N^6-H of amino OMe^{6}A [which is located predominantly anti relative to the ring N(1)]. By contrast, there is no analogous proton exchange between the cytidine amino protons and the N(1)-H of imino OMe^{6}A with which it associates. These processes are accompanied by two other exchange phenomena, viz., hindered rotation of the cytidine amino group and tautomeric exchange in OMe^{6}A. The latter process, involving proton transfer between N(1)-H and N^6-H of the two tautomers, is concentration-dependent, suggesting that such exchange is the result of formation of complexes between the amino and imino species. Addition of cytidine and resultant proton exchange between C and imino OMe^{6}A accelerates the process of tautomeric exchange, best placed in evidence at −40 °C, where the constants k_{AI} and k_{IA} increase from about 10^{-4}–10^{-5} to 0.2–0.3 s^{-1}. Both exchange processes are consequently coupled. Clarification of the mechanism involved and the transition forms of the participating molecules is, however, rendered difficult by the manner of transfer of the proton from the N^6-H of amino OMe^{6}A to the NH_2 of cytidine, which does not interact directly with the former by association.

Whatever the type of complex between imino OMe^{6}A and C, the mechanism of proton transfer probably proceeds via some transient forms, perhaps the rare imino species of cytidine and/or its protonated form or the protonated forms of OMe^{6}A. Participation of rare tautomeric species and of protonated bases in proton exchange processes in A:U and G:C base pairs in solution has been postulated by Iwahashi and Kyogoku (1980). But studies on tautomerism in the gas phase (Beak, 1977; Nowak et al., 1980; Shugar & Szczepaniak, 1981), as well as indirect measurement of the difference in enthalpy between the keto and enol forms of 1-substituted uracil (Beak & White, 1982), argue against existence of the rare tautomeric forms. By contrast, X-ray diffraction studies of short DNA fragments demonstrate stabilization of the double-helical structure, with mismatched bases, via hydrogen bonding with adenine protonated at N(1) (Hunter et al., 1986).

Notwithstanding the foregoing difficulties in accounting for the precise mechanism of the shift in tautomeric equilibrium of OMe^{6}A, it is clear that selective base pairing of the imino tautomer of this promutagen with cytidine and the previously

noted selective base pairing of its amino form with uridine (Stolarski et al., 1984) are fundamental to an understanding of the molecular mechanism of hydroxylamine and methoxyamine mutagenesis.

The imino tautomer may behave like guanosine during transcription on templates containing OMe^6A residues, and the effect of the incoming potentially complementary base residue on the population of the two tautomeric forms will be reflected in both the frequency and type of mutations. It is also clear that N^6-hydroxy- or N^6-methoxyadenosines would be extremely useful models for studies on site-directed mutagenesis, as succesfully applied some time ago by Flavell et al. (1974) with N^4-hydroxycytidine.

Proton exchange involving N-H protons of the bases, at the level of the double helix in aqueous medium, has been observed directly by means of the transfer saturation method (Johnston & Radfield, 1977). Such exchange is a reflection of the local relaxation of the double-helical structure (so-called "breathing" modes) leading to exposure of base N-H protons to the aqueous environment. It is highly likely that, during local structural transformations of nucleic acids in the course of transcription and in mutagenic processes, the proton exchange phenomena described here and by Iwahashi and Kyogoku (1980) may be involved.

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Registry No. C, 65-46-3; OMe^6A, 19399-25-8; NH_2OH, 7803-49-8; NH_2OMe, 67-62-9.

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