Hydroxylamine and methoxyamine mutagenesis: Displacement of the tautomeric equilibrium of the promutagen N6-methoxyadenosine by complementary base pairing

ARTICLE *in* BIOCHEMISTRY · JULY 1984

Impact Factor: 3.02 · Source: PubMed

4 AUTHORS, INCLUDING:

Borys Kierdaszuk
University of Warsaw
77 PUBLICATIONS **1,086** CITATIONS
SEE PROFILE

David Shugar
Polish Academy of Sciences
408 PUBLICATIONS **6,880** CITATIONS
SEE PROFILE

Hydroxylamine and Methoxyamine Mutagenesis: Displacement of the Tautomeric Equilibrium of the Promutagen N^6-Methoxyadenosine by Complementary Base Pairing†

Ryszard Stolarski, Borys Kierdaszuk, Curt-Eric Hagberg, and David Shugar*

ABSTRACT: The imino–amino tautomeric equilibrium of the promutagenic adenosine analogue N^6-methoxy-2′,3′,5′-tri-*O*-methyladenosine [$OMe^6A(Me)_3$], in solvents of various polarities, has been studied with the aid of ^1H and ^{13}C NMR spectroscopy. The high energy barrier (free enthalpy $\Delta G^{\ddagger} = 80 \pm 5$ kJ·mol^{-1}) between the two tautomeric species renders possible direct observation of the independent sets of all ^1H and ^{13}C signals from each of them. The equilibrium ranges from 10% imino in CCl_4 to 90% in aqueous medium. Thermodynamic parameters, including energy barriers and lifetimes, were calculated from the temperature dependence of the equilibrium. Essentially similar results prevail for the promutagenic N^6-hydroxy analogue. The conformations of the sugar moieties, and of the base about the glycosidic bond, for both tautomers are similar to those for adenosine. The conformation of the exocyclic N^6-OCH_3 group, which determines the ability of each species to form planar associates (hydrogen-bonded base pairs), has also been evaluated. Formation of autoassociates of $OMe^6A(Me)_3$ and of heteroassociates with the potentially complementary 2′,3′,5′-tri-*O*-methyluridine and -cytidine, in chloroform solution, was also investigated. The amino form base pairs with uridine and the imino form with cytidine. Formation of a complementary base pair by a given tautomeric species was accompanied by an increase of up to 10% in the population of this species and a concomitant decrease in population of the other species. With respect to the conformation of the exocyclic N^6-OCH_3 group, appropriate structures are presented for both auto- and heteroassociates. The overall findings are examined in relation to hydroxylamine mutagenesis, possible tautomerism of nonmodified bases, and transcription involving these.

Initial interpretations of the mutagenic activities of hydroxylamine (NH_2OH) and methoxyamine (NH_2OCH_3) were based on conversion of cytosine and 5-methylcytosine residues in DNA to the corresponding N^4-hydroxy and N^4-methoxy derivatives (Phillips & Brown, 1967; Janion & Shugar, 1965a,b; Kochetkov & Budowsky, 1969). The subsequent demonstration that these mutagens also react with adenosine residues, albeit more slowly, to give the N^6-hydroxy- and N^6-methoxyadenosines [for review see Budowsky (1976)] suggested the need for an investigation of the tautomerism and base pairing properties of these analogues. This is further indicated by the fact that N^6-hydroxyadenine is a promutagenic analogue in the rII mutant system of phage T4 (Freese, 1968), in which it behaves like adenine or guanine, and that in an in vitro transcription system, N^6-methoxyadenosine residues paired with both U and C,[1] but not A and G (Singer & Spengler, 1982); i.e., these residues exhibit dual functional properties in base pairing, like N^4-hydroxycytidine residues (Budowsky et al., 1971a; Flavell et al., 1974), which exist in aqueous medium as a mixture of two tautomers (Brown et al., 1968; Sverdlov et al., 1971).

However, as in the case of N^4-hydroxycytosines, or methoxycytosines, the base pairing properties of the two tautomeric forms of N^6-hydroxyadenosines, or methoxyadenosines, will also depend on the orientation of the N^6-OH or N^6-OCH_3 groups, which may be syn or anti to the ring N(1), a problem discussed in detail elsewhere (Shugar et al., 1976; Birnbaum et al., 1979; Kierdaszuk & Shugar, 1983), and to which we revert to below.

It was originally noted, with the aid of UV and IR spectroscopy, that the tautomerism of the foregoing analogues (Morozov et al., 1982) was appreciably dependent on solvent medium (Kierdaszuk et al., 1983a, 1984). Subsequent preliminary experiments demonstrated that the tautomeric equilibrium of N^6-methoxy-2′,3′,5′-tri-*O*-methyladenosine [$OMe^6A(Me)_3$][1] was shifted on addition of the potentially complementary uridine (Kierdaszuk et al., 1983a) and prompted us to undertake a more detailed investigation of this phenomenon (which, to our knowledge, has not been hitherto observed), largely with the aid of NMR spectroscopy.

Materials and Methods

Previously described procedures were employed for the syntheses of $OMe^6A(Me)_3$ (Budowsky et al., 1971b; Kierdaszuk et al., 1984) and $U(Me)_3$ and $C(Me)_3$ (Kuśmierek et al., 1973).

Solvents for NMR spectroscopy included C^2HCl_3 (99.5 mol % ^2H), ^2H_2O (99.9 mol % ^2H), $C^2H_3O^2H$ (99.0 mol % ^2H), and $(C^2H_3)_2SO$ (99.8 mol % ^2H), all from Merck (Darmstadt, GFR), and CCl_4 from POCH (Gliwice, Poland).

The ^1H NMR spectra were recorded mainly on Bruker 360 FT and Jeol XL 200 instruments. A Jeol-JNM-4H-100 cw spectrometer was employed to record the spectra of $OMe^6A(Me)_3$ at a concentration of 0.2 M in mixtures of C^2HCl_3 and

† From the Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 02-089 Warszawa, Poland (R.S., B.K., and D.S.), the Research Laboratories, Astra Lakemedel AB, S-15185 Södertälje, Sweden (C.-E.H.), and the Institute of Biochemistry and Biophysics, Academy of Sciences, 02-532 Warszawa, Poland (D.S.). *Received September 7, 1983.* This work was supported by the Polish Academy of Sciences (Project 09.7.1), the Polish National Cancer Research Program (Project PR-6), and the Polish Ministry of Sciences, Higher Education and Technology (Project MR I.5).

* Address correspondence to this author at the Department of Biophysics, Institute of Experimental Physics, University of Warsaw, 02-089 Warszawa, Poland.

[1] Abbreviations: $OMe^6A(Me)_3$, N^6-methoxy-2′,3′,5′-tri-*O*-methyladenosine; $OH^6A(Me)_3$, N^6-hydroxy-2′,3′,5′-tri-*O*-methyladenosine; $U(Me)_3$, 2′,3′,5′-tri-*O*-methyluridine; $C(Me)_3$, 2′,3′,5′-tri-*O*-methylcytidine; A, adenosine; G, guanosine; C, cytidine; U, uridine; dA, 2′-deoxyadenosine; dC, 2′-deoxycytidine; dU, 2′-deoxyuridine; $(Me)_4Si$, tetramethylsilane; Me_2SO, dimethyl sulfoxide.

0006-2960/84/0423-2906$01.50/0

Table I: Proton and Carbon Chemical Shifts (in ppm) vs. Internal $(Me)_4Si$ of $OMe^6A(Me)_3$ in $(C^2H_3)_2SO$ and in C^2HCl_3 at a Concentration of 0.2 M at 30 °C

tautomeric form	NH	H(2)	H(8)	H(1')	H(2')	H(3')	H(4')	H(5')	H(5'')	N^6-OCH_3	5',2',3'-OCH_3[a]		
						$(C^2H_3)_2SO$							
amino	11.00	8.34	8.42	6.07	4.54	4.10	4.19	3.62	3.56	3.79	3.40	3.37	3.33
imino	11.25	7.61	8.05	5.88	4.43	4.04	4.15	3.58	3.52	3.77	3.39	3.35	3.33
						C^2HCl_3							
amino	10.45	8.41	8.53	6.26	4.30	4.09	4.31	3.82	3.64	4.01	3.60	3.49	3.47
imino	9.86	7.69	7.97	6.07	4.20	4.05	4.26	3.74	3.60	3.90	3.52	3.47	3.44

tautomeric form	C(2)	C(4)	C(5)	C(6)	C(8)	C(1')	C(2')	C(3')	C(4')	C(5')	N^6-OCH_3	5',2',3'-OCH_3[a]		
							C^2HCl_3							
amino	152.9	150.0	118.8	155.7	140.9	87.1	81.3	77.9	82.5	71.5	64.8	59.3	58.6	58.2
imino	142.7	140.4	119.6	141.2	136.4	86.7	81.3	78.2	83.0	71.8	61.7	59.3	58.6	58.2

[a] Assignment of the signals due to 5'-OCH_3 is correct. It was not possible to unequivocally distinguish between 2'-OCH_3 and 3'-OCH_3.

Table II: Values of Proton-Proton Vicinal Coupling Constants and of Some of the Carbon-Proton[a] Coupling Constants for $OMe^6A(Me)_3$ in Dimethyl Sulfoxide and Chloroform Solutions and Calculated Principal Conformational Parameters of the Sugar Ring[b]

tautomeric form	J(1,2)	J(1',2')	J(2',3')	J(3',4')	J(4',5')	J(4',5'')	J(5',5'')	C(2')-endo (%)	5'-CH_2OH *gg* (%)
				$(C^2H_3)_2SO$					
amino		5.2	4.8	4.2	4.0	4.8	−10.9	55	41
imino	3.6	5.6	4.8	3.9	4.0	4.8	−10.9	59	41
				C^2HCl_3					
amino		3.0	4.8	6.3	2.6	2.7	−10.9	33	84
imino	3.5	3.6	5.0	6.0	2.8	3.2	−10.9	38	75

tautomeric form	J(2,2)	J(8,8)	J(5,6)	J(5,1)	J(8,1')	J(1',1')	J(2',2')	J(3',3')	J(4',4')	J(5',5')
					C^2HCl_3					
amino	203.9	214.8	1.5		3.7	168.5	148.9	147[c]	150.1	135.5
imino	205.1	214.8		4.5	3.7	167.2	148.5	147[c]	150.2	141.0

[a] For the carbon-proton coupling constants J(C,H), the first figure in the parentheses refers to the carbon nucleus and the second to the proton coupled to it. [b] Conditions as in Table I. [c] Approximate value only, because of overlapping of signals.

CCl_4, as well as in $C^2H_3O^2H$, and the temperature-dependent spectra of $C(Me)_3$ and its mixtures with $OMe^6A(Me)_3$ at concentrations of 0.2 M.

The ^{13}C NMR spectra were run on a Jeol XL 200 at a frequency of 50 MHz.

Proton and carbon chemical shifts were determined relative to internal $(Me)_4Si$ with accuracies of ±0.01 and ±0.05 ppm, respectively. Proton–proton coupling constants are accurate to ±0.1 Hz and proton–carbon coupling constants to ±1 Hz.

In some instances, because of solubility considerations and other technical problems, it proved necessary to revert to data obtained with the aid of UV and IR spectroscopy. The latter results, described in detail in a separate communication (Kierdaszuk et al., 1984), will be referred to in the present text where necessary.

Results and Discussion

Tautomeric Equilibrium of $OMe^6A(Me)_3$. The ^1H and ^{13}C NMR spectra of $OMe^6A(Me)_3$, in C^2HCl_3 and in $(C^2H_3)_2SO$, exhibit a characteristic doubling of all signals, with identical ratios of relative integral intensities in each solvent. A typical ^1H spectrum has been presented elsewhere (Kierdaszuk et al., 1983a), and a corresponding typical ^{13}C spectrum is exhibited in Figure 1. However, the ratios of relative integral intensities of the two sets of signals differ with the nature of the solvent (Tables I and II). It should be noted that doubling of signals embraces not only the heterocyclic ring atoms but also those of the carbohydrate moiety. This points to the existence of the compound in solution as an equilibrium mixture of two

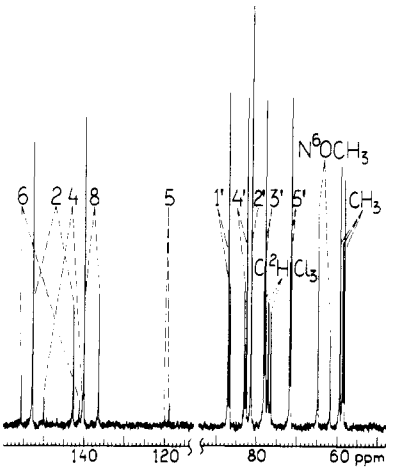
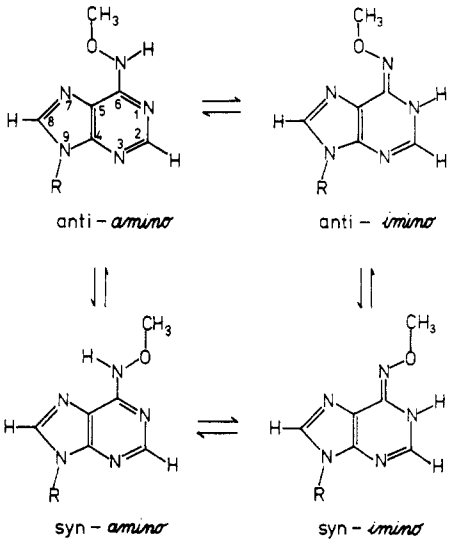


FIGURE 1: ^{13}C NMR spectrum of $OMe^6A(Me)_3$, 0.2 M in C^2HCl_3. The numbers correspond to the various carbon atoms of the molecule. The higher signal for each pair corresponds to the amino form and the lower to the imino form. Chemical shifts are relative to internal $(Me)_4Si$.

forms with comparable energies, the two being separated by a relatively high energy barrier, which accounts for the slow exchange between them on the NMR time scale at room temperature. For a solution in $(C^2H_3)_2SO$, an increase in temperature to 80 °C led to broadening or overlapping of the individual pairs of signals (depending on the differences in chemical shifts), testifying to an increase in the rate of exchange between them to a level corresponding to the so-called

Scheme I: Amino-Imino Tautomeric Equilibrium and Syn-Anti Rotamer Equilibrium of the Exocyclic N^6-OMe Group Relative to the Ring N(1), in $OMe^6A(Me)_3$ (R = 2′,3′,5′-Tri-*O*-methylribose) or OMe^6A (R = Ribose)



intermediate rate of exchange on the NMR time scale (Gutowsky et al., 1953).

Analyses of the ^1H and ^{13}C spectra permitted identification of the two species as the amino and imino tautomers (see Scheme I), further confirmed independently with the aid of UV and IR spectroscopy (Kierdaszuk et al., 1984). Existence of the imino form, with the mobile proton on adenine ring N(1), is corroborated by the coupling constant of 3.5 Hz between H(2) and the mobile proton on N(1). This interpretation is not entirely unambiguous, inasmuch as the mobile proton may theoretically be located equally at N(3); the resulting system of proton–proton and proton–carbon couplings would be quite similar because of the low magnitudes of the couplings between a carbon and a mobile proton separated from it by two bonds. The values for the two cases are below the limits of resolution of the instrument and lead only to some broadening of the carbon signals. By contrast, a good criterion for establishment of the structure of the imino form is provided by a comparison of the ratios of the heights of the signals of C(6) to C(4) for the amino and imino forms. These ratios are identical, thus confirming the location of the mobile proton at N(1) in the imino form, as shown in Scheme I. Otherwise, the coupling of N(1)-H with C(6) would be absent and replaced by coupling of N(3)-H with C(4), with an accompanying change in heights of both carbon signals to give a higher ratio of their heights for the imino, than for the amino, form.

Tautomer Populations and Thermodynamic Parameters. The populations of the two tautomeric forms, in several different solvents, were determined from the ^1H signal intensities and are listed in Table III. It is clear that the equilibrium populations are markedly dependent on solvent polarity, with a preference for the amino form in nonpolar or low polarity solvents. The population of the amino form in chloroform, at a concentration of 0.2 M, is 70%. Stepwise replacement of solvent by CCl_4 leads to an increase in the proportion of the amino form, to attain a value of 80% in a 50% (v/v) solution of CCl_4 in chloroform; simultaneously the proportion of the imino form decreases correspondingly. Measurements with higher proportions of CCl_4 were precluded by limited solubility of the compound, but IR spectroscopy in 100% CCl_4 demonstrated the presence of at least 90% of the amino form (Kierdaszuk et al., 1984). In polar solvents such as dimethyl sulfoxide and methanol, it is the imino form that predominates,

Table III: Populations of the Amino and Imino Tautomers of $OMe^6A(Me)_3$ in Various Solvents, at Two Concentrations and Different Temperatures, in the Presence and Absence of the Potentially Complementary $U(Me)_3$ at an Equimolar Concentration

solvent	concn (M)	temp (°C)	population (%)[a] amino	imino
^2H_2O	0.2	+30	10[b]	90[b]
$C^2H_3O^2H$	0.2	+30	20[c]	80[c]
	0.2	−30	10[c]	90[c]
$(C^2H_3)_2SO$	0.2	+30	22	78
	0.2	+80	31	69
C^2HCl_3	0.2	+30	70	30
	0.2	−30	78	22
	0.04	+30	67	33
	0.04	−30	76	24
$+U(Me)_3$	0.2	+30	79	21
	0.04	+30	75	25
% CCl_4 in C^2HCl_3				
33	0.2	+30	76	24
40	0.2	+30	79	21
50	0.2	+30	80	20

[a] Estimated accuracy ±2%. [b] Based on infrared spectroscopy, to an accuracy of ±5% [see Kierdaszuk et al. (1984)]. [c] From spectra run on a Jeol JNM 4H-100 cw instrument, hence somewhat less accurate, ±10%.

its proportion attaining a value of about 90% in the most polar solvent, water. The latter value, derived from analysis of the ^1H spectrum, is only a rough approximation because of the single set of signals observed in aqueous medium, where the rate of exchange between the tautomeric forms is rapid (see below). This value is, however, independently corroborated by the results of aqueous IR spectroscopy (Kierdaszuk et al., 1984).

In dimethyl sulfoxide at 80 °C, it proved feasible to evaluate the mean lifetime, 2τ, of the tautomers and the mean rate of exchange between them, $1/(2\tau)$, either from coalescence of the ^1H signals of the 5′-OCH_3 (difference in chemical shifts 5.4 Hz at 30 °C) or from the broadening of the N^6-OCH_3 signals (difference in chemical shifts 12.8 Hz at 30 °C) under conditions of slow exchange on the NMR time scale, close to the intermediate rate, where the signals coalesce (Gutowsky et al., 1953). The results in both instances are similar, with $1/(2\tau) = 11.5\ s^{-1}$.

From the broadening of the N^6-OCH_3 signals and the population values, one may evaluate the lifetimes τ(imino) and τ(amino) of each form and the rates of exchange in either direction, viz., $1/\tau(\text{imino}) = 7.5\ s^{-1}$ and $1/\tau(\text{amino}) = 15.5\ s^{-1}$. Furthermore, the Eyring equations

$$1/(2\tau) = kT/h \exp[-\Delta G^{\ddagger}/(RT)]$$

$$1/\tau(\text{amino}) = kT/h \exp[-\Delta G_a^{\ddagger}/(RT)]$$

$$1/\tau(\text{imino}) = kT/h \exp[-\Delta G_i^{\ddagger}/(RT)]$$

give the mean free enthalpy for the barrier between the two forms at 80 °C, $\Delta G^{\ddagger} = 80 \pm 5\ kJ \cdot mol^{-1}$, and of the barrier for conversion of the amino form to the imino and conversely, $\Delta G_a^{\ddagger}$ and $\Delta G_i^{\ddagger}$, respectively. The difference $\Delta G_i^{\ddagger} - \Delta G_a^{\ddagger} = 2.2\ kJ \cdot mol^{-1}$ is the difference in free enthalpies of the energy minima, $\Delta G°$, for the two tautomers, in agreement with the value $\Delta G° = 2.4 \pm 0.2\ kJ \cdot mol^{-1}$, determined independently from the equation

$$\Delta G° = RT \ln (P_{\text{imino}}/P_{\text{amino}})$$

where the P's refer to populations. In methanol, which has an exchangeable proton (deuteron), the energy barrier is reduced to about $65\ kJ \cdot mol^{-1}$, probably due to the facilitated transfer of the proton via the solvent. In aqueous medium

there is an even more profound decrease in the barrier between the two forms, so that the resulting increase in rate of exchange leads to a single set of averaged signals in the NMR spectrum. Since the populations of the tautomers were determined at only two temperatures, the differences in enthalpy, $\Delta H°$, and entropy, $\Delta S°$, for the energy minima in dimethyl sulfoxide and chloroform ($\sim$8 kJ·mol^{-1} and $\sim$0.02 kJ·mol^{-1}·deg^{-1}; $\sim$3.5 kJ·mol^{-1} and $\sim$0.002 kJ·mol^{-1}·deg^{-1}) are only approximate.

Conformation of the Exocyclic N^6-OCH_3 Group. A key factor in the ability of each of the tautomeric forms of $OMe^6A(Me)_3$ to form hydrogen-bonded planar autoassociates, or heteroassociates with the potentially complementary uridine and cytidine, is the conformation of the exocyclic N^6-OCH_3 group. The structure of such associates will be dependent on the orientation of this group, which may be syn or anti with respect to the ring N(1), as shown in Scheme I and discussed in detail elsewhere (Shugar et al., 1976; Kierdaszuk et al., 1983a, 1984). If the orientation of this group is syn, then the N^6 of the imino form would be unable to function as an acceptor in hydrogen bonding, while the N^6-H of the amino form would be incapable of acting as a donor in Watson–Crick base pairing, although it could still participate in Hoogsteen type pairing.

The foregoing situation is well illustrated by the behavior of 1-methyl-N^4-methoxycytosine which, in chloroform solution, is predominantly in the imino form with the N^4-OCH_3 group syn with respect to the ring N(3). The compound cannot, therefore, participate in Watson–Crick base pairing with the potentially complementary 9-substituted adenines. The two do, however, form a heteroassociate via inverse Watson–Crick base pairing, with the N(1)-H as donor and the C(2)=O as acceptor (Kierdaszuk & Shugar, 1983; Kierdaszuk et al., 1983b).

In fact, for the amino form of $OMe^6A(Me)_3$ in chloroform, the low value of the coupling constant between C(5) and N^6-H, $\leq$2 Hz, points to a preference for the rotamer syn, with a consequent *cis*oidal location of the N^6-H and C(5) (Scheme I). If the N^6-H were *trans* to C(5), one would anticipate a coupling constant of about 4.5 Hz such as occurs between N(1)-H and C(5) in the imino tautomer. In chloroform solution, a decrease in temperature to −30 °C did not produce any modification such as might be expected if there were an equilibrium mixture of two rotamers. It is, however, not possible to exclude the presence of some proportion of the minor rotamer (see discussion on heteroassociation with cytidine).

For the corresponding imino tautomer, the ^{1}H and ^{13}C spectra do not furnish the necessary parameters for defining the orientation of the N^6-OCH_3 group. There are, however, indications that the rotamer orientation is predominantly, if not exclusively, syn. For 1-methyl-N^4-methoxycytosine in chloroform, only the syn rotamer of the N^4-OCH_3 group is observed (Kierdaszuk & Shugar, 1983); in the solid state there is an intramolecular hydrogen bond between the N(3)-H and the oxygen of the exocyclic group (Shugar et al., 1976; Birnbaum et al., 1979), which probably contributes to stabilization of the form syn in the low-polar chloroform solution. An analogous stabilizing effect may be anticipated for the imino form of $OMe^6A(Me)_3$ via an intramolecular hydrogen bond between the N(1)-H and the oxygen of the N^6-OCH_3 group.

Molecular Conformations of Tautomers. Like other purine nucleosides, both tautomeric forms of $OMe^6A(Me)_3$ exhibit a dynamic equilibrium N $\rightleftharpoons$ S, or C(2′)-endo $\rightleftharpoons$ C(3′)-endo, for the two conformers of the sugar ring, with comparable populations of the two forms in polar solvents and a preference (60–70%) for the form S in chloroform (Table II). The pseudorotation parameters, determined graphically (Guschlbauer, 1980), were similar, with $\tau_m = 41°$, $^NP = 7°$, and $^SP = 173°$. This is in accord with earlier findings that methylation of sugar hydroxyls only minimally affects the conformation of nucleosides (Ekiel et al., 1978).

The exocyclic 5′-CH_2OCH_3 side chain of each form exhibits the usual dynamic equilibrium of three classical rotamers, gauche–gauche (*gg*), gauche–trans (*gt*), and trans–gauche (*tg*). In aqueous medium and in Me_2SO, there is a 40–50% preference for the *gg* rotamer, which increases to more than 80% in a low polarity solvent like chloroform (see Table II).

The chemical shifts of the sugar protons, in particular H(2′), indicate that the amino form of $OMe^6A(Me)_3$ in dimethyl sulfoxide shows up as an equilibrium mixture of comparable populations of the syn and anti conformers about the glycosidic bond, as in the case of adenosine (Stolarski et al., 1980). The difference in chemical shifts between the H(2′) of $OMe^6A(Me)_3$ and that of adenosine, 0.2 ppm, is due simply to the influence of the *O*-methyl groups in the former: the H(2′) is shifted −0.3 ppm by the 2′-OCH_3 and +0.1 ppm by the 3′-OCH_3. The identical coupling constant between H(1′) and C(8) in both tautomers, 3.7 Hz, further implies that they possess similar conformations about the glycosidic bond.

Tautomerism and Conformation of $OH^6A(Me)_3$. N^6-Hydroxyadenosine, the product of reaction of adenosine with the mutagen hydroxylamine, is also a promutagenic base analogue. The ^{1}H NMR spectrum of $OH^6A(Me)_3$ in dimethyl sulfoxide likewise exhibited the existence of two tautomeric species, amino and imino, separated by a relatively high energy barrier, with $\Delta G^‡ \approx 80$ kJ/mol at 25 °C, as for $OMe^6A(Me)_3$. The populations of the imino and amino forms were approximately 75% and 25%, respectively, hence similar to the populations of the tautomeric species of $OMe^6A(Me)_3$. As for the latter, the population of the imino form of $OH^6A(Me)_3$ increases to more than 90% in methanol solution. Additional observations demonstrated that the conformational parameters of the two tautomeric species did not differ significantly from those for the N^6-methoxy analogue, and further studies were therefore confined to the latter, additionally facilitated by its higher solubility in low-polar solvents.

Effect of Planar Association (Base Pairing) on Tautomeric Equilibrium. In solvents of low polarity such as chloroform, $OMe^6A(Me)_3$ forms planar autoassociates via hydrogen bonding (Kierdaszuk et al., 1984). Autoassociation may be followed by the changes in chemical shifts of the protons directly involved in formation of hydrogen bonds with changes in concentration and temperature. An increase in concentration from 0.04 to 0.2 M is accompanied by characteristic deshielding of the N^6-H of the amino form by 1.37 ppm and of the N(1)-H of the imino form by 0.72 ppm. At a concentration of 0.2 M, a decrease in temperature from +30 to −30 °C leads to deshielding of the same protons by 1.48 and 0.42 ppm. From Table III it will be seen that an increase in concentration and/or a decrease in temperature, factors which promote increased autoassociation (as well as heteroassociation with potentially complementary analogues), is accompanied by a minor shift in the tautomeric equilibrium in favor of the amino form.

Addition to a chloroform solution of $OMe^6A(Me)_3$ of an equimolar concentration of the potentially complementary $U(Me)_3$ provokes analogous changes in chemical shifts of the N^6-H of the amino form, by 0.68 ppm in a 0.04 M solution and by 0.10 ppm in a 0.2 M solution. By contrast, there is

Table IV: Influence of Change in Concentration from 0.04 to 0.2 M on Changes in ^{13}C Chemical Shifts for $OMe^6A(Me)_3$, for $U(Me)_3$ (Corresponding to Formation of Autoassociates of Each), and for an Equimolar Mixture of the Two (Formation of Heteroassociates)[a]

carbon atom	tautomeric form	δ(free, 0.2 M) − δ(free, 0.04 M)[b]	δ(mixt) − δ(free)[c] 0.2 M	δ(mixt) − δ(free)[c] 0.04 M	δ(mixt, 0.2 M) − δ(mixt, 0.04 M)[d]
$OMe^6A(Me)_3$					
C(2)	amino	0.13	0.17	0.20	0.10
	imino	0.61	0.14	0.10	0.65
C(4)	amino	−0.14	−0.25	−0.22	−0.17
	imino	−0.1[e]	0.07	−0.1[d]	0.05[e]
C(5)	amino	−0.27	−0.80	−0.61	−0.46
	imino	−0.09	−0.10	*f*	*f*
C(6)	amino	−0.07	−0.51	−0.36	−0.22
	imino	0.17	0.03	0.03	0.17
C(8)	amino	−0.18	−0.01	−0.07	−0.12
	imino	−0.05	−0.02	0.00	−0.07
$U(Me)_3$					
C(2)		0.30	0.36	0.32	0.34
C(4)		0.68	0.39	0.51	0.56
C(5)		0.02	0.11	−0.07	0.06
C(6)		0.05	−0.07	−0.05	0.03

[a] All solutions in C^2HCl_3 at 30 °C. [b] Difference in chemical shifts for each nucleoside at 0.2 and 0.04 M. [c] Differences in chemical shifts for one nucleoside in the presence and absence of an equimolar amount of the complementary nucleoside, at concentrations of 0.2 and 0.04 M. [d] Differences in chemical shifts for equimolar mixtures of the two complementary nucleosides at two concentrations. [e] Approximate value, since the C(4) signal is obscured by C(8). [f] The C(5) signal of the imino form is below background.

no significant change in chemical shift (<0.1 ppm) of the N(1)-H of the imino form at either concentration. This implies formation of strong heteroassociates between $U(Me)_3$ and the amino form of $OMe^6A(Me)_3$ with the proviso that, at high concentrations, autoassociation is a more effective competing process. On the other hand, the imino form does not detectably base pair with $U(Me)_3$.

Hence, formation of auto- and heteroassociates influences the tautomeric equilibrium of $OMe^6A(Me)_3$, shifting it in the direction of the amino form. The increase in population of the amino form is particularly marked on formation of heteroassociates with $U(Me)_3$ (9%, with an equal decrease in population of the imino form; see Table III). But this effect is also present with autoassociation (3–4%), to an extent probably greater than for the imino form.

An analogous situation prevails on addition of an equimolar amount of $C(Me)_3$ to a chloroform solution of $OMe^6A(Me)_3$. This results in deshielding of the N(1)-H of the imino form of the latter, by 0.46 ppm at 0.04 M and 0.68 ppm at 0.2 M. There is no significant change in chemical shift of the N^6-H of the amino form (see further discussion below). Clearly defined base pairing consequently occurs between $C(Me)_3$ and only the imino form of $OMe^6A(Me)_3$, and this is accompanied by a 10% increase in population of this form, at the expense of the amino species.

Recapitulating, formation by each tautomer of $OMe^6A(Me)_3$ of a planar heteroassociate (base pair) with the potentially complementary base is accompanied by a shift in the tautomeric equilibrium in favor of this tautomer. Such shifts in tautomeric equilibrium as a result of base pairing are of obvious biological significance (see below).

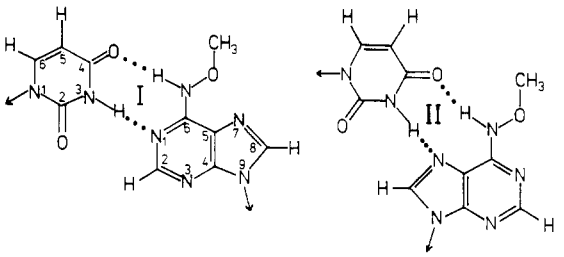
Autoassociation and Heteroassociation with U(Me)$_3$ from ^{13}C Chemical Shifts. ^{13}C chemical shifts have been widely applied to studies of planar associates, in media of low polarity, of 9-ethyladenine with various analogues of 1-cyclohexyluracil (Iwahashi & Kyogoku, 1977) and of $A(Me)_3$ with 1-substituted N^4-methoxycytosines (Kierdaszuk & Shugar, 1983). Appreciable, unequivocally identifiable, chemical shifts are observed for the C=O carbons, because of involvement of the oxygens as hydrogen acceptors in stabilization of the planar complexes.

For $U(Me)_3$ in chloroform, an increase in concentration from 0.04 to 0.2 M leads to characteristic deshielding of C(2) and C(4) by 0.30 and 0.68 ppm, respectively (Table IV), pointing to formation of two types of autoassociates, as for 1-cyclohexyluracil (Iwahashi & Kyogoku, 1977). Addition, at each concentration, of an equimolar amount of the potentially complementary $OMe^6A(Me)_3$ provokes analogous deshieldings of both C(2) and C(4) of $U(Me)_3$ by 0.32–0.56 ppm (Table IV). Hence, $U(Me)_3$ forms heteroassociates of the Watson–Crick type via C(4)=O, as well as "reversed" Watson–Crick pairing via C(2)=O. For both auto- and heteroassociates, pairing via C(4)=O appears to predominate.

On formation of autoassociates, or heteroassociates with $U(Me)_3$, the changes in chemical shifts of the carbon atoms of $OMe^6A(Me)_3$ point to formation of planar complexes but, unfortunately, do not permit unambiguous determination of the structures of these complexes (Charts I and II). A similar situation prevails for associates of 9-substituted adenines (Iwahashi & Kyogoku, 1977). With the imino form, there is strong deshielding of C(2) by 0.61 ppm and C(6) by 0.17 ppm. With the amino form, C(5) is shielded by 0.27 ppm and C(8) by 0.18 ppm, while C(2) is deshielded by 0.13 ppm. The changes in chemical shifts of other carbons do not exceed 0.1 ppm (Table IV). Addition of $U(Me)_3$ at either concentration results in only minimal changes in chemical shifts of the imino form (<0.1 ppm), whereas pronounced changes are observed for the amino form, viz., deshielding of C(5) and C(6) by up to 0.80 and 0.51 ppm at a concentration of 0.2 M, and changes of about half this magnitude for C(4), which is shielded, and C(2), which is deshielded. These changes, while similar to those accompanying a change in concentration, are even more clear-cut.

The ^{13}C NMR spectra consequently confirm the results obtained by ^1H NMR spectroscopy that both tautomeric species of $OMe^6A(Me)_3$ form autoassociates while only the amino tautomer forms heteroassociates with uridine. It is pertinent to note that the ^{13}C chemical shift changes accompanying formation of complexes involve both deshielding (carbonyl carbons) and shielding effects. An analogous deshielding effect has been noted for the C(5) of adenine on formation of a complex of 9-ethyladenine with 1-cyclohexyluracil (Iwahashi & Kyogoku, 1977). Pairing of $OMe^6A(Me)_3$ with $U(Me)_3$ is also accompanied by shielding of C(6) of $OMe^6A(Me)_3$, as against deshielding of this carbon in 9-ethyladenine when paired with 1-cyclohexyluracil.

Chart I: Possible Structures of Hydrogen-Bonded Planar Heteroassociates of the Amino Form of $OMe^6A(Me)_3$ with $U(Me)_3$, via the Uracil C(4)=O[a]



[a] Similar complexes are possible via the uracil C(2)=O (see text).

Furthermore, pairing of 9-ethyladenine with 1-cyclohexyluracil does not affect the chemical shifts of C(2) and C(8), despite formation of hydrogen bonds via the adenine N(1) (Watson–Crick pairing), as well as N(7) (Hoogsteen pairing). With $OMe^6A(Me)_3$, shifts of C(2) and C(8) are observed on pairing with $U(Me)_3$, but they are significantly lower than the changes in shifts of C(5) and C(6), whereas autoassociation of the imino form is accompanied by a marked change in the shift of C(2), with much smaller changes in shifts of C(5) and C(6). The most plausible interpretation of the foregoing, as suggested elsewhere (Iwahashi & Kyogoku, 1977), is the anisotropic effect of the complementary base in the complex, which contributes to changes in magnetic environment of the carbons following formation of hydrogen bonds.

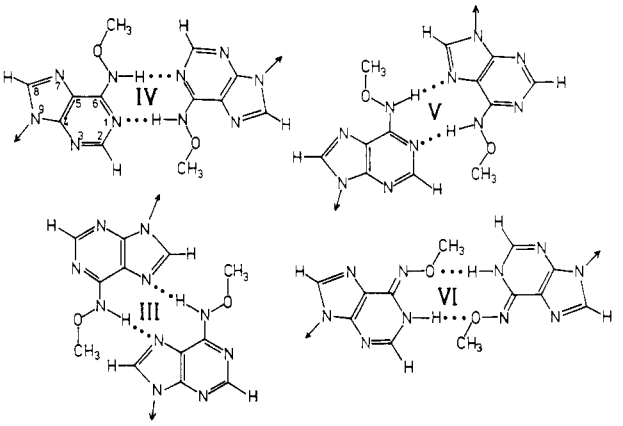
For the amino form of $OMe^6A(Me)_3$, with the exocyclic N^6-OCH_3 rotamer anti to N(1), complex formation with $U(Me)_3$ may occur via N^6-H and N(1), i.e., Watson–Crick pairing (I, Chart I), and with N^6-OCH_3 as the syn rotamer, Hoogsteen type pairing is feasible via N^6-H and N(7) (II, Chart I). Although, as discussed above (in relation to the conformation of the exocyclic group), the latter appears the more likely, the available data do not unequivocally exclude involvement of the former.

An analogous situation holds for autoassociates. With the amino form, autoassociates hydrogen bonded via N^6-H and N(7) (III, Chart II) should predominate, but the ^1H and ^{13}C NMR results do not unequivocally exclude such structures as IV and V (Chart II). With the imino form, and the syn rotamer of the N^6-OCH_3 group, autoassociates are possible via N(1)-H and the oxygen of the exocyclic group (VI, Chart II). This is the most likely structure, testifed to by the marked change in chemical shift of C(2) (Table IV), as well as of H(2) (0.13 ppm on increasing the concentration from 0.04 to 0.2 M), the latter being the most pronounced observed effect on a proton not directly participating in hydrogen bonding. Such marked changes in chemical shifts are probably due to the anisotropic influence of the N^6-OMe group which, in this type of autoassociate, is located in the vicinity of C(2) and H(2). More direct evidence for the foregoing proposed structures would require the use of ^{15}N NMR spectroscopy.

Association of $OMe^6A(Me)_3$ with $C(Me)_3$. Attempts to follow possible formation of associates of $OMe^6A(Me)_3$ with the potentially complementary $C(Me)_3$, by means of ^1H and ^{13}C NMR spectroscopy, proved somewhat more difficult because of the presence of supplementary exchange effects (see below).

At concentrations of 0.04 and 0.2 M in chloroform at 30 °C, the amino protons of $C(Me)_3$ are not visible, even in the presence of an equimolar concentration of $OMe^6A(Me)_3$. This is clearly due to the strong broadening of these signals as a result of hindered rotation of the amino group about the

Chart II: Possible Structures of Hydrogen-Bonded Planar Autoassociates of $OMe^6A(Me)_3$



C(4)–N^4 bond, i.e., coalescence under conditions of exchange at an intermediate rate on the NMR time scale relative to the difference in chemical shifts of the two protons. This effect was previously noted for 1-methylcytosine in dimethyl sulfoxide (Becker et al., 1965), dimethylformamide (Shoup et al., 1971), and a 1:1 mixture of dimethylformamide and dimethyl sulfoxide (Shoup et al., 1966), as well as for 5′-CMP in neutral aqueous medium (Raszka & Kaplan, 1972).

This phenomenon was further examined by following the temperature dependence of the ^1H NMR spectrum of a 0.2 M solution of $C(Me)_3$ in chloroform. At 0 °C the two amino protons were revealed as strong broad signals with equal integral intensities, and at −10 °C these signals were clearly defined at 6.83 and 9.01 ppm. A similar effect was observed in the temperature-dependent spectra of a 1:1 mixture of $OMe^6A(Me)_3$ and $C(Me)_3$, the chemical shifts of the amino protons being 6.63 and 8.52 ppm at −10 °C and 6.87 and 8.76 ppm at −30 °C.

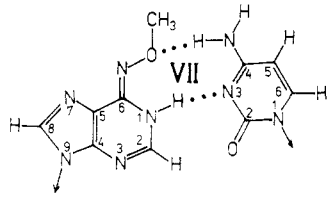
An additional characteristic feature was the broadening of the ^1H and ^{13}C signals of both tautomeric forms of $OMe^6A(Me)_3$ at a concentration of 0.2 M at 30 °C, followed by addition of an equimolar amount of $C(Me)_3$. Broadening of the signals was considerably reduced by lowering the concentration to 0.04 M or by decreasing the temperature. Furthermore, with a 1:1 mixture, at concentrations of 0.2 or 0.04 M, at 30 °C, the N^6-H signal of the amino form of $OMe^6A(Me)_3$ vanishes, whereas the N(1)-H of the imino form at 9.60 (0.04 M) and 10.54 ppm (0.2 M) is present. Reduction of the temperature to −30 °C led to appearance, and stepwise sharpening, of the N^6-H located at 11.71 ppm at −30 °C, as compared to 11.93 ppm in the absence of $C(Me)_3$.

A similar behavior is exhibited by the signal of the N^6-OCH_3 protons in a 1:1 mixture at a concentration of 0.2 M. By contrast, when the concentration of the mixture is reduced to 0.04 M, this signal is already visible at 30 °C.

The foregoing data testify to the pronounced influence of association between $C(Me)_3$ and $OMe^6A(Me)_3$ (discussed further, below) on the tautomeric equilibrium and conformation of the exocyclic N^6-OMe group, of the latter. An additional effect which may possibly be involved is the proposed exchange of protons between cytidine and guanosine base paired at the monomer level (Iwahashi & Kyogoku, 1980) and in tRNA (Rüterjans et al., 1982).

In distinct contrast to association with $U(Me)_3$ (see above), only the imino species of $OMe^6A(Me)_3$ strongly associates with $C(Me)_3$ under the same experimental conditions. This is reflected by the marked deshielding of the N(1)-H of the imino tautomer, in the presence of $C(Me)_3$, by 0.46 ppm at a con-

Chart III: Experimentally Determined Structure of the Planar Heteroassociate of the Imino Form of $OMe^6A(Me)_3$ with $C(Me)_3$



centration of 0.04 M, increasing to 0.68 ppm at 0.2 M and to 1.02 ppm on reducing the temperature to −30 °C. Pronounced deshielding, in the 1:1 mixture, is also exhibited by C(2), viz., 0.84 ppm at a concentration of 0.2 M and 0.59 ppm at 0.04 M, and by H(2), 0.17 ppm at 0.2 M and 0.10 ppm at 0.04 M. The striking effect on C(2), also reflected in the deshielding of C(2) by 0.6 ppm on formation of autoassociates (Table IV), is most likely due to the anisotropic effect of the closely proximate C(2)=O of $C(Me)_3$ on association of the latter with $OMe^6A(Me)_3$ via the hydrogen bonds N(1)–H···N(3) and N^6–O···H–N^6, as shown in Chart III.

It should also be emphasized that addition of $C(Me)_3$ to $OMe^6A(Me)_3$ does not significantly affect the proton or carbon chemical shifts of the amino form of the latter. This is particularly evident for N^6-H at −30 °C, under which conditions it is clearly visible, and for C(5) and C(6), the signals of which are significantly shifted on association with $U(Me)_3$. It follows that the amino tautomer associates weakly, if at all, with $C(Me)_3$, notwithstanding that it could by inverse Watson–Crick pairing, which is sterically permissible.

Concluding Remarks. The observed hydrogen-bonded base pairing of $C(Me)_3$ and $U(Me)_3$ with the imino and amino forms, respectively, of $OMe^6A(Me)_3$ and the shifts in tautomeric equilibrium accompanying such base pairing, as well as the influence of solvent on the tautomeric equilibrium, clearly contribute to an understanding of the dual functional properties of N^6-methoxyadenine (and of N^6-hydroxyadenine) both in vitro (Budowsky et al., 1971a; Flavell et al., 1974; Singer & Spengler, 1982) and in vivo (Freese, 1968; Budowsky et al., 1975). In the system of phage T4 rII mutants (Freese, 1968; Budowsky et al., 1975) they behave like A or G, producing A → G type transitions. This is possible because of the complementarity between the amino tautomer and U and the imino tautomer with C, as observed in this study at the monomer level.

In the transcription of synthetic matrices consisting of copolymers of dC and N^6-methoxy-2′-deoxyadenosine, where incorporation of U is reported to be 10-fold higher than C (Singer & Spengler, 1982), the behavior of the N^6-methoxyadenine residues is similar with regard to dual complementarity. The results obtained in the present investigation show additionally that, at the monomer level, there may be Watson–Crick base pairing between the amino and imino forms of N^6-methoxyadenine with U and C in the template during transcription. However, at the monomer level, where there is a preference for the syn rotamer of the N^6-methoxy group, a Hoogsteen type base pairing predominates between the amino tautomer and U (II, Chart I) as for 1-substituted uracil and 9-substituted adenine (Watanabe et al., 1981). There is no direct evidence as to whether this occurs at the level of transcription, but such base pairing has been observed in triple helices of poly(U) and poly(A) (Arnott & Bond, 1973) and poly(dT) and poly(dA) (Arnott & Selsing, 1974).

Although attention has previously been drawn to this problem (Shugar & Szczepaniak, 1981), the present findings constitute, to our knowledge, the first direct demonstration of a constrained shift in the tautomeric equilibrium of a purine or pyrimidine analogue under the influence of base pairing. This phenomenon is, on the other hand, by no means unique and has been previously observed with other classes of compounds. A recent striking illustration is the very large shift in the tautomeric equilibrium of the imidazole ring of a histidyl residue in α-lytic protease, attributed to a hydrogen-bonded interaction between the unusual N(3)-H form of the imidazole ring and an adjacent carboxylate group of aspartic acid (Kanamori & Roberts, 1983).

A similar situation is encountered with N^4-hydroxy- and N^4-methoxycytidines, detectable only in the imino form in low-polar solvents (Brown et al., 1968), in the gas phase (Kulinska et al., 1980), and in the solid state (Shugar et al., 1976; Birnbaum et al., 1979). The imino form, although predominant in polar media like water, is reported to be in equilibrium with from 10% (Brown et al., 1968) to 25% (Sverdlov et al., 1971) of the amino species, and this is reflected, as in the case of N^6-methoxyadenosine, by its dual functional properties in polymerase systems where, both as a free triphosphate substrate or when located in a template (Budowsky et al., 1971b; Flavell et al., 1974), it comports itself like uridine or cytidine. However, at the monomer level in chloroform solution, it does not form Watson–Crick base pairs with either adenosine or guanosine because of the syn conformation, relative to the ring N(3), of the exocyclic N^4-hydroxy (or methoxy) group (Kierdaszuk & Shugar, 1983a). It does, however, form an inverse Watson–Crick pair with adenosine, under which conditions there is no steric hindrance by the exocyclic group (Kierdaszuk et al., 1983b). Its behavior in polymerase systems must therefore be due to modification by the enzymes of the exocyclic group conformation and, at least to some extent, of the tautomeric equilibrium (Spengler & Singer, 1981).

It appears reasonable to inquire as to whether such constrained shifts in tautomeric equilibrium are possible in the case of natural purines and pyrimidines found in nucleic acids. At the monomer level, i.e., nucleoside triphosphates, new experimental pieces of evidence based in large part on studies of tautomerism in the gas phase (Beak, 1977; Shugar & Szczepaniak, 1981) argue against the existence of presumed enol and imino forms of the bases. This argument is strengthened by the recent indirect experimental determination by Beak & White (1982) of the enthalpy difference between the keto form of uracil and its 4-enol tautomer, about 80 kJ/mol, in the vapor phase. This large enthalpic driving force in favor of the keto tautomer is further enhanced by hydrogen-bonding effects in solution (Beak & White, 1983).

The situation is somewhat different at the level of a DNA helix. It was proposed some time ago on the basis of theoretical considerations (Löwdin, 1963) that a hydrogen involved in one of the base pair hydrogen bonds may undergo transfer, by a tunneling mechanism, from one base to its complementary residue, thus leading to a change in tautomerism of both bases. This would, in fact, constitute an example of a constrained shift in tautomeric equilibrium. Although initially attractive as a source of spontaneous mutagenesis, it is still rather controversial.

Registry No. $OMe^6A(Me)_3$, 87079-99-0; $OH^6A(Me)_3$, 89437-76-3; $U(Me)_3$, 39848-57-2; Me_2SO, 67-68-5; CCl_4, 56-23-5; H_2O, 7732-18-5; methanol, 67-56-1; chloroform, 67-66-3.

References

Arnott, S., & Bond, P. (1973) *Nature (London), New Biol. 244*, 99–101.

Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 509–521.

Beak, P. (1977) *Acc. Chem. Res. 10*, 186–192.
Beak, P., & White, J. M. (1982) *J. Am. Chem. Soc. 104*, 7073–7077.
Becker, E. D., Miles, H. T., & Bradley, R. B. (1965) *J. Am. Chem. Soc. 87*, 5575–5582.
Birnbaum, G. I., Kulikowski, T., & Shugar, D. (1979) *Can. J. Biochem. 57*, 308–313.
Brown, D. M., Hewlins, M. J. E., & Schell, P. (1968) *J. Chem. Soc. C*, 1925–1929.
Budowsky, E. I. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 16*, 125–188.
Budowsky, E. I., Sverdlov, E. D., & Spasokukotskaya, T. N. (1971a) *FEBS Lett. 17*, 336–338.
Budowsky, E. I., Sverdlov, E. D., & Monastyrskaya, G. S. (1971b) *Biochim. Biophys. Acta 246*, 320–328.
Ekiel, I., Darzynkiewicz, E., Dudycz, L., & Shugar, D. (1978) *Biochemistry 17*, 1530–1536.
Flavell, R. A., Sabo, D. L., Bundle, E. F., & Weissman, C. (1974) *J. Mol. Biol. 89*, 255–272.
Freese, E. B. (1968) *Mutat. Res. 5*, 299–301.
Guschlbauer, W. (1980) *Biochim. Biophys. Acta 610*, 47–55.
Gutowsky, H. S., McCall, D. M., & Slichter, C. P. (1953) *J. Chem. Phys. 21*, 279–285.
Iwahashi, H., & Kyogoku, Y. (1977) *J. Am. Chem. Soc. 99*, 7761–7765.
Iwahashi, H., & Kyogoku, Y. (1980) *J. Am. Chem. Soc. 102*, 2913–2917.
Janion, C., & Shugar, D. (1965a) *Biochem. Biophys. Res. Commun. 18*, 617–622.
Janion, C., & Shugar, D. (1965b) *Acta Biochim. Pol. 12*, 337–355.
Kanamori, K., & Roberts, J. D. (1983) *Acc. Chem. Res. 16*, 35–41.
Kierdaszuk, B., & Shugar, D. (1983) *Biophys. Chem. 17*, 285–295.
Kierdaszuk, B., Stolarski, R., & Shugar, D. (1983a) *FEBS Lett. 158*, 128–130.
Kierdaszuk, B., Stolarski, R., & Shugar, D. (1983b) *Eur. J. Biochem. 130*, 559–564.
Kierdaszuk, B., Stolarski, R., & Shugar, D. (1984) *Acta Biochim. Pol.* (in press).
Kochetkov, N. K., & Budowsky, E. I. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 403–437.
Kulińska, K., Psoda, A., & Shugar, D. (1980) *Acta Biochim. Pol. 27*, 57–65.
Kuśmierek, J. T., Giziewicz, J., & Shugar, D. (1973) *Biochemistry 12*, 194–199.
Löwdin, P.-O. (1963) *Rev. Mod. Phys. 35*, 724–729.
Morozov, Yu. V., Savin, F. A., Chekhov, V. O., Budowsky, E. I., & Yakovlev, D. Yu. (1982) *J. Photochem. 20*, 229–252.
Phillips, H. H., & Brown, D. M. (1967) *Prog. Nucleic Acid Res. Mol. Biol. 7*, 349–368.
Raszka, M., & Kaplan, N. O. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2025–2029.
Rüterjans, H., Kaun, E., Hull, W. E., & Limbach, H. H. (1982) *Nucleic Acids Res. 10*, 7027–7039.
Shoup, R. R., Miles, H., & Becker, E. D. (1966) *Biochem. Biophys. Res. Commun. 23*, 194–201.
Shoup, R. R., Becker, E. D., Miles, H. T. (1971) *Biochem. Biophys. Res. Commun. 43*, 1350–1353.
Shugar, D., & Szczepaniak, K. (1981) *Int. J. Quantum Chem. 20*, 573–578.
Shugar, D., Huber, C. P., & Birnbaum, G. I. (1976) *Biochim. Biophys. Acta 447*, 274–284.
Singer, B., & Spengler, S. (1982) *FEBS Lett. 139*, 69–75.
Spengler, S., & Singer, B. (1981) *Biochemistry 20*, 7290–7294.
Stolarski, R., Dudycz, L., & Shugar, D. (1980) *Eur. J. Biochem. 108*, 111–121.
Sverdlov, E. D., Krapivko, A. P., & Budowsky, E. I. (1971) *Khim. Geterotsikl. Soedin. 9*, 1264–1267.
Watanabe, M., Sugeta, H., Iwahashi, H., Kyogoku, Y., & Kainosho, M. (1981) *Eur. J. Biochem. 117*, 553–558.