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Kinetics of Hydrogen–Deuterium Exchange in Guanosine 5′-Monophosphate and Guanosine 3′:5′-Monophosphate Determined by Laser-Raman Spectroscopy†

M. J. Lane and G. J. Thomas, Jr.*

ABSTRACT: Pseudo-first-order rate constants governing the deuterium exchange of 8-CH groups in guanosine 5′-monophosphate (5′-rGMP) and guanosine 3′:5′-monophosphate (cGMP) were determined as a function of temperature in the range 30–80 °C by means of laser-Raman spectroscopy. For each guanine nucleotide the logarithm of the rate constant exhibits a strictly linear dependence on reciprocal temperature: i.e., $k_\psi = Ae^{-E_a/RT}$ with $A = 8.84 \times 10^{14}$ h^{-1} and $E_a = 24.6$ kcal/mol for 5′-rGMP and $A = 3.33 \times 10^{13}$ h^{-1} and $E_a = 22.2$ kcal/mol for cGMP. Exchange of the 8-CH groups in guanine nucleotides is generally 2–3 times more rapid than in adenine nucleotides [cf. G. J. Thomas, Jr., & J. Livramento (1975) *Biochemistry 14*, 5210–5218]. As in the case of adenine nucleotides, cyclic and 5′ nucleotides of guanine exchange at markedly different rates at lower temperatures, with exchange in the cyclic nucleotide being the more facile. Each of the guanine nucleotides was prepared in four different isotopic modifications for Raman spectral analysis. The Raman frequency shifts resulting from the various isotopic substitutions have been tabulated, and assignments have been given for most of the observed vibrational frequencies.

In previous work from this laboratory (Thomas & Livramento, 1975) laser-Raman spectroscopy was used to determine the pseudo-first-order rate constant (k_ψ) governing the exchange in neutral D_2O solution of hydrogen by deuterium at the position 8 carbon of the adenine ring. Values of k_ψ, accurate to within 5%, were determined over the temperature range 30–90 °C for adenosine 5′-monophosphate (5′-rAMP), adenosine 3′:5′-monophosphate (cAMP), and polyriboadenylic acid [poly(rA)].

The plot of k_ψ vs. the reciprocal temperature was strictly linear only for 5′-rAMP in the cited temperature interval. Above 60 °C exchange in cAMP approached that of 5′-rAMP. Below 50 °C, however, exchange in cAMP was considerably faster than in 5′-rAMP. The exchange kinetics thus indicate that below 50 °C the nucleotides 5′-rAMP and cAMP are electronically dissimilar or are subject to associative interactions which differ in such a way that the lability of the 8-CH bond is significantly affected. It was not clear from the information obtained previously (Thomas & Livramento, 1975) whether 5′-rAMP should be regarded as the norm and cAMP as anomalously rapid with respect to 8-CH exchange or, conversely, whether exchange in 5′-rAMP is likely to have been retarded due to associative interactions, e.g., base stacking. Exchange in poly(rA) was found to be greatly retarded compared with either of the adenine nucleotides, a result which is expected in view of the polynucleotide secondary structure.

In order to gain further insight into the role of the cyclic ribosyl phosphate moiety on the kinetics of 8-CH exchange in the attached purine and to complete a systematic study of exchange in the common purine ribonucleotides, we have investigated the rates of deuterium exchange of 8-CH groups in guanosine 5′-monophosphate (5′-rGMP) and guanosine 3′:5′-monophosphate (cGMP). Quantitative Raman spectroscopy was again used to determine both the exchange rate constants over the temperature range 30–80 °C and the related Arrhenius activation energy, E_a, and frequency factor, A, in the expression shown in eq 1.

$$\ln k_\psi = \ln A - E_a/RT \quad (1)$$

The results obtained here are compared with those obtained previously on corresponding adenine nucleotides to reveal the extent to which different substituents at the C(2) and C(6) purinic ring positions affect the lability of the 8-CH group in the imidazole heterocycle. Analogous rate constant determinations were not made for poly(rG) because of the well-known aggregation of this polynucleotide and the problems

† From the Department of Chemistry, Southeastern Massachusetts University, North Dartmouth, Massachusetts 02747. *Received February 15, 1979.* This paper is Part 18 in the series Raman Spectral Studies of Nucleic Acids. Support of the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service Grant AI 11855-04, is gratefully acknowledged.

0006-2960/79/0418-3839$01.00/0

attendant with sample handling at the concentrations required for Raman spectroscopy.

Selective deuteration of the 8-CH groups of 5′-rGMP and cGMP also provides new spectroscopic information which permits refinement of earlier vibrational Raman assignments to frequencies of specific subgroups of the guanine nucleotides (Lord & Thomas, 1967; Small & Peticolas, 1971; Tsuboi et al., 1973).

Isotopic hydrogen exchange at the C(8) position of purines has been known for many years (Eidinoff & Knoll, 1953) and has been investigated previously by a number of physicochemical methods other than laser-Raman spectroscopy. Among these are proton magnetic resonance spectroscopy (Schweizer et al., 1964), infrared spectroscopy (Fritzsche, 1967), and radioassay of tritium-labeled derivatives (Tomasz et al., 1972; Lesnik et al., 1973; Elvidge et al., 1974; Gamble et al., 1976). The last mentioned method, which involves monitoring the rate of tritiation or radiolabeling, has gained the widest use, apparently because the tritium-labeled purines can be detected in very dilute solutions. On the other hand, Raman spectroscopy has certain advantages over tritium labeling, particularly for applications to nucleic acids which contain different bases or are easily hydrolyzed. Among these advantages are the following: (1) the Raman spectrum simultaneously monitors *both* the structural integrity of the molecule *and* the concentration of isotopically exchanged species; (2) the Raman spectrum is capable, in principle, of discriminating simultaneous exchange in different purines of a polynucleotide or oligonucleotide; e.g., exchange of adenine and guanine residues can be monitored separately in RNA by use of the Raman spectrum; and (3) procedures of Raman spectroscopy are relatively simple vis-à-vis the labeling and assaying procedures involved in the tritiation studies. In short, the Raman method offers advantages of simplicity, speed, and versatility. On the other hand, the Raman spectroscopic method as employed here requires a much greater quantity of material than is required for the tritiation technique.

Experimental Procedures

5′-rGMP and cGMP (sodium salts; Sigma Chemical) were dissolved to 0.075 M at pH 7.0 in H_2O or at pD 7.4 in D_2O (99.8% deuterium; Aldrich Chemical). Solution pH or pD was adjusted as required by addition of NaOH or NaOD (Aldrich Chemical). No gel formation was encountered with either nucleotide. In neutral H_2O or D_2O solution, appreciable hydrogen-bonding interaction between the guanylic residues is not expected to occur at the concentrations and temperatures employed in this work (Gellert et al., 1962; Miles & Frazier, 1972; Pinnavaia et al., 1975). Further discussion of this point will be given below.

Details of sample handling, with regard to both incubations for isotope exchange and procedures of Raman spectroscopy, are as described previously (Thomas & Livramento, 1975). In addition, the back-exchange (hydrogen for deuterium) of deuterated 5′-rGMP and cGMP was achieved by incubation of the 8-CD form of the nucleotide in H_2O solution. To isolate each deuterated nucleotide as a solid, the fully deuterated sample was lyophilized from D_2O solution. Deuterium contamination of H_2O solutions, as well as hydrogen contamination of D_2O solutions, was kept below the minimum level readily detectable by Raman spectroscopy, viz., 2 mol %.

Raman spectra were recorded on a Spex Ramalog spectrometer using 488.0-nm excitation from a Coherent CR-2 argon ion laser. The radiant power at the sample was in the range 200–300 mW. Further details of Raman instrumentation are given elsewhere (Thomas & Barylski, 1970; Medeiros & Thomas, 1971).

Total nucleotide concentrations were determined precisely by diluting aliquots of the stock solutions 1:1000 in phosphate pH 7.5 buffer and recording UV absorption spectra on a Beckman Model DK-2A spectrometer.

pH and pD (=pH + 0.4) measurements were made on a Beckman Expandomatic SS-2 meter with a Fisher microelectrode.

Concentrations of 8-CH and 8-CD forms of guanine nucleotides in D_2O solution are proportional to the intensities of the 1480- and 1458-cm^{-1} lines, respectively, in the Raman spectrum. The rate of exchange may therefore be determined by either the rate of intensity decay at 1480 cm^{-1} or the rate of intensity increase at 1458 cm^{-1}. These two independent methods of calculating k_ψ provide results which are in close agreement with one another, but the former is considered to be more accurate, i.e., subject to uncertainties of less than 5%, as explained previously (Thomas & Livramento, 1975). The Raman lines of the guanine nucleotides at 1480 and 1458 cm^{-1} correspond, respectively, to the lines of the adenine nucleotides at 1485 and 1462 cm^{-1}.

Intense Raman lines of the phosphate groups, which are unaffected by deuterium substitutions in the guanine ring (or ribose moiety), are used as internal standards for quantifying the 1480-cm^{-1} intensity in terms of molecular concentration. The lines are centered at ca. 980 (5′-rGMP) and 1088 (cGMP) cm^{-1}. Furthermore, since all spectra are recorded at the same temperature (10 °C) and pH (7.0), the phosphate line intensities are not subject to changes in effective phosphate ion concentration in a given run. It may be added that the phosphate line intensities will not vary significantly even should variations of temperature or pH (within a few tenths of a pH unit) occur. The 980-cm^{-1} line is assigned to the symmetric PO_3^{2-} stretching vibration in 5′-rGMP and the 1088-cm^{-1} line to the symmetric PO_2^- stretching vibration in cGMP (Thomas & Hartman, 1973).

As shown previously (Thomas & Livramento, 1975), k_ψ is evaluated from the slope of a plot of the left side of eq 2 vs.

$$\ln \frac{[C_H{}^0]}{[C_H]} = \ln \frac{I_{1480}{}^0 - I_{1480}{}^\infty}{I_{1480}{}^t - I_{1480}{}^\infty} = k_\psi t \qquad (2)$$

t, where $[C_H]/[C_H{}^0]$ is the fraction of nucleotide remaining nonexchanged at time t and $I_{1480}{}^t$ is the Raman intensity at 1480 cm^{-1} after exchange time t, normalized to the intensity of the internal standard. Raman intensities were determined by measuring the peak height above a base line tangent to the wings of the peak in question. This method provided results which were within experimental error of those obtained by an alternative but more time-consuming band area method. Arrhenius parameters and rate constants were evaluated by linear regressions of eq 1 and 2. In each determination of k_ψ, two or more runs were carried out on independently prepared samples which were handled with identical protocol.

Results

The nucleotides 5′-rGMP and cGMP can be conveniently studied in four different isotopic forms, structures I–IV, where R is the appropriate ribosyl phosphate moiety. The conversion I → II occurs immediately upon contact of I with D_2O solvent. II → III ("forward exchange") is governed by k_ψ and requires prolonged exposure of II to D_2O. III can be isolated as a solid by lyophilization from D_2O. III → IV occurs immediately upon contact of III with H_2O solvent. Finally, IV → I ("back exchange") is also governed by k_ψ and requires prolonged exposure of IV to H_2O.

Raman spectra of aqueous solutions of 5′-rGMP and cGMP

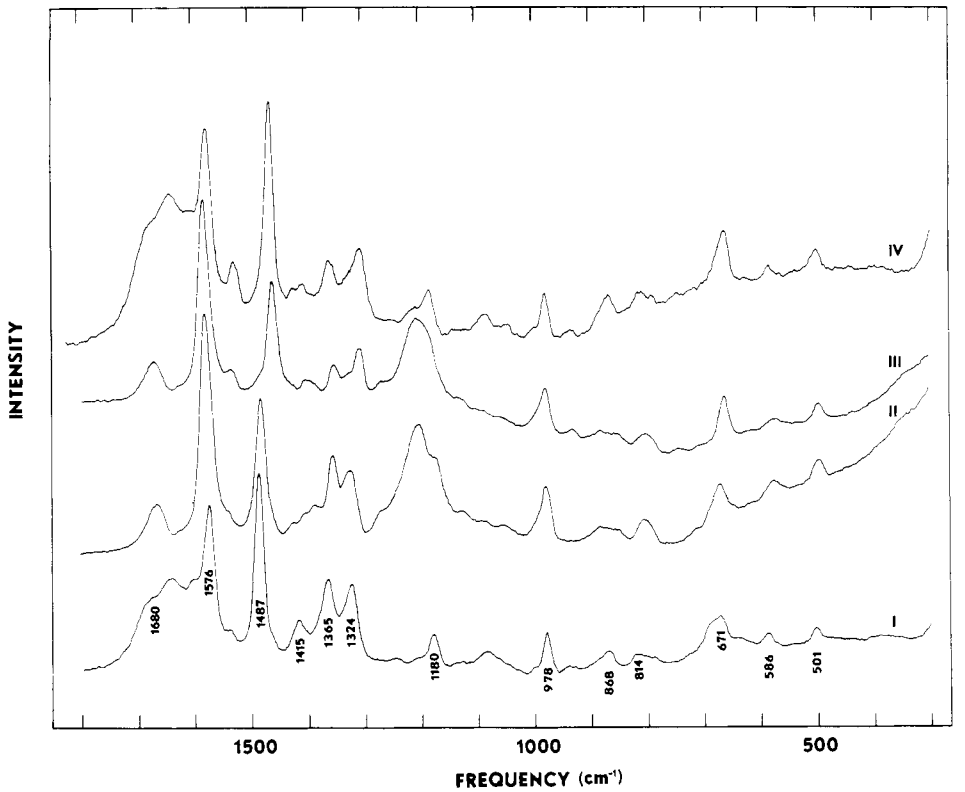
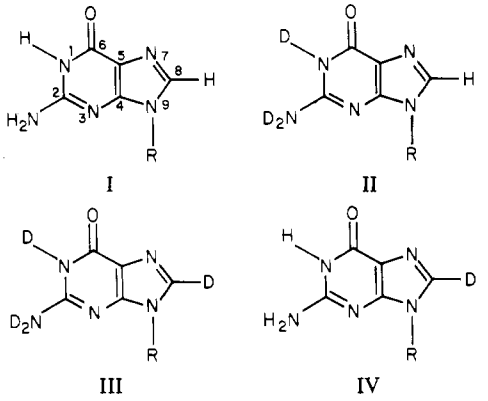


FIGURE 1: Raman spectra in the 300–1800-cm^{-1} region of 5′-rGMP in four isotopic modifications (see text). Nucleotide concentration was 0.075 M in H_2O (pH 7.0) or D_2O (pD 7.4). Instrument conditions: wavelength of excitation λ = 488.0 nm, spectral slit width $\Delta\sigma$ = 8 cm^{-1}, rise time τ = 3 s, scan speed s = 50 cm^{-1}/min, sample temperature t = 10 °C.



in the four isotopic modifications are shown in Figures 1–3. Raman frequencies are listed in Table I. Figure 4 illustrates the pseudo-first-order kinetics at 80 °C of forward exchange in both 5′-rGMP and cGMP. Rate constants evaluated from the data of Figure 1 by use of eq 2 and from similar data obtained at other temperatures are tabulated in Table II. These results yield the Arrhenius parameters of Table II by use of eq 1 and Figure 5.

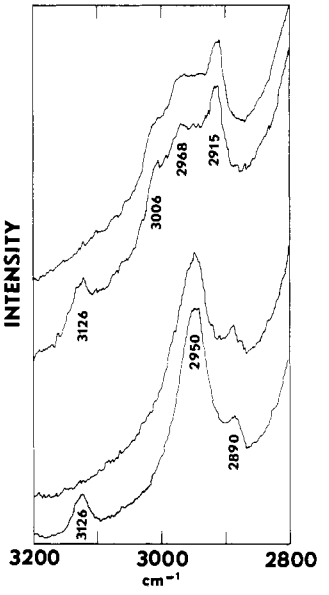


FIGURE 2: Raman spectra of 5′-rGMP and cGMP in the CH stretching region. Other conditions are as in Figure 1, except τ = 10 s and s = 25 cm^{-1}/min.

Discussion

Assignment of the Raman Frequencies. Raman frequencies of aqueous 5′-rGMP have been discussed previously (Lord & Thomas, 1967; Small & Peticolas, 1971; Rice et al., 1973). No comparable studies have been made of cGMP, although Forrest & Lord (1977) have assigned the oxygen–phosphorus stretching vibrations of the cyclic phosphate ester in cGMP, in accord with assignments for cAMP (Thomas & Hartman, 1973; Thomas & Livramento, 1975).

In Table I the Raman frequencies of both 5′-rGMP and cGMP are listed for each of the four isotopic modifications I–IV. Vibrational frequencies of structures III and IV, which have not been reported previously, provide isotope-shift information that is helpful in characterizing guanine ring modes. The following rationale was used in arriving at the assignments listed in the last column of Table I.

The Raman frequencies were classified into three main groups, viz., frequencies common to both 5′-rGMP and cGMP, frequencies specific to 5′-rGMP, and frequencies specific to cGMP. Each group was further subdivided into frequencies common to both H_2O and D_2O solutions of the nucleotides, frequencies specific to H_2O solutions only, and frequencies specific to D_2O solutions only. Frequencies common to both 5′-rGMP and cGMP are assigned with confidence to the guanine ring, those of 5′-rGMP to the ribose 5′-phosphate

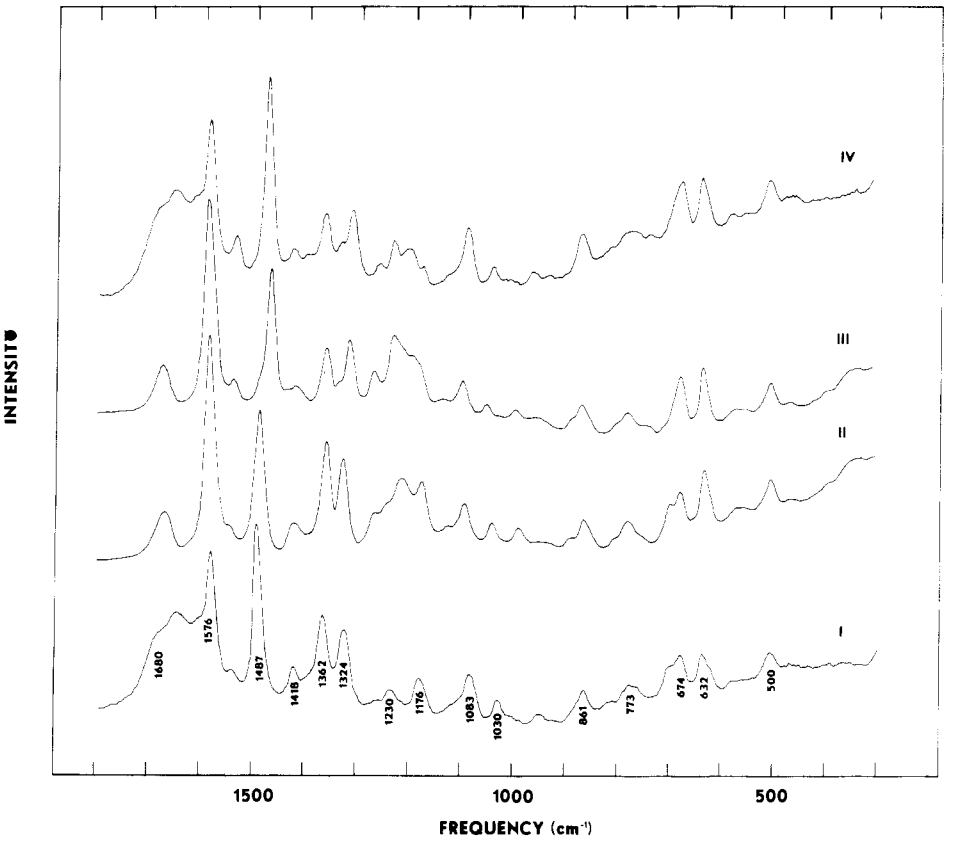


FIGURE 3: Raman spectra in the 300–1800-cm^{-1} region of cGMP in four isotopic modifications (see text). Conditions are as in Figure 1.

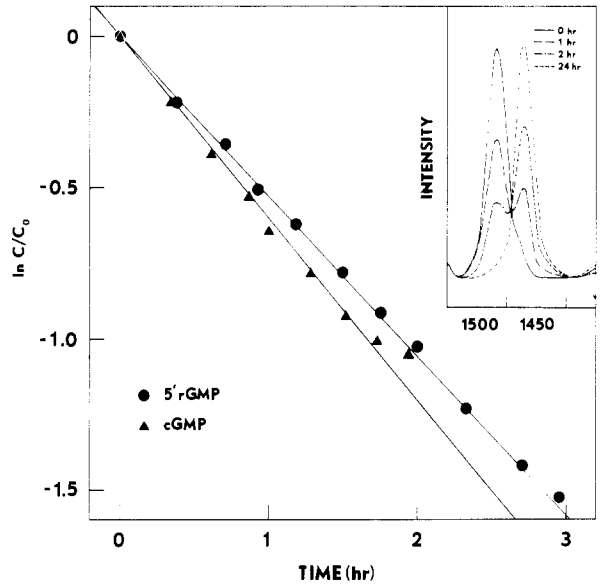


FIGURE 4: Semilogarithmic plots of the relative concentration of nonexchanged guanine nucleotide vs. the time of incubation at 80 °C in D_2O: (●) 5′-rGMP, (▲) cGMP. The insert shows the observed Raman line intensities at 1480 and 1458 cm^{-1} at various stages of incubation of 5′-rGMP at 80 °C.

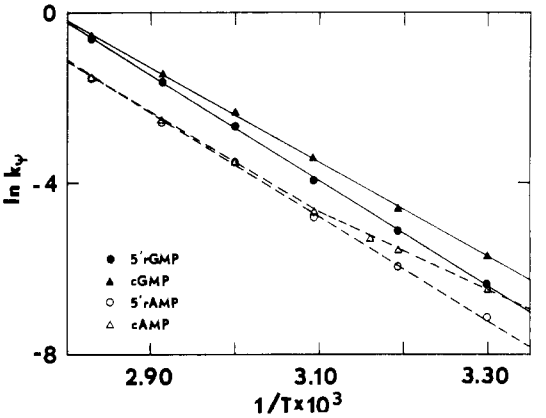


FIGURE 5: Plots of ln k_ψ vs. $1/T$ for 5′-rGMP (●) and cGMP (▲) obtained in the present study and for 5′-rAMP (○) and cAMP (△) obtained by Thomas & Livramento (1975). Each linear plot is a least-squares fit to the data points shown.

residue, and those of cGMP to the cyclic 3′:5′-phosphate residue. Moreover, depending upon whether the frequencies occur for H_2O solutions, D_2O solutions, or both, they can be categorized principally as ring (or skeletal) modes, as vibrations involving attached or exchangeable ring substituents, or as coupled vibrations involving both the ring skeleton and external substituents. The assignments are further substantiated by reference to previously published spectra of nucleotide derivatives. For example, frequencies specific to the cyclic 3′:5′-phosphate residue must also meet the criterion of being present in Raman spectra of cAMP (Thomas & Livramento, 1975). We note also that certain Raman lines are restricted to either the 8-CH form or the 8-CD form of the guanine ring, irrespective of the solvent. Such Raman lines are duly noted in the assignments of Table I.

In the foregoing, no Raman frequency is declared common to two or more members (i.e., columns) of Table I unless the following conditions are met. First, the average of the frequencies must not deviate by more than 1% from its member frequencies; and second, the Raman intensity must not vary by more than 50% between members. For example, the markedly different Raman intensities observed at ~855 cm^{-1} for 5′-rGMP and at ~861 cm^{-1} for cGMP suggest that the frequencies are due to vibrations of different types (Table I).

Where reliable assignments have been made previously for vibrational Raman and/or infrared frequencies of guanine nucleotides (Lord & Thomas, 1967; Miles & Frazier, 1972; Howard & Miles, 1965; Tsuboi et al., 1973), the assignments

Table I: Raman Frequencies and Assignments of 5′-rGMP and cGMP in Aqueous Solutions[a]

structure I (H_2O)		structure II (D_2O)		structure III (D_2O)		structure IV (H_2O)		
5′-rGMP-d_0	cGMP-d_0	5′-rGMP-d_3	cGMP-d_3	5′-rGMP-d_4	cGMP-d_4	5′-rGMP-d_1	cGMP-d_1	assignment
	460 (0)		460 (0)		460 (0)		460 (0)	cyclic ester ring def
501 (1)	500 (1)	495 (1)	495 (1)	495 (1)	495 (1)	500 (1)	500 (2)	G ring def, cyclic ester ring def
	575 (0)		565 (0, b)		565 (0, b)		575 (0)	cyclic ester ring def
586 (1)		576 (0)		574 (0)		584 (0)		G ring def, 5′-rP
	625 (s)						625 (s)	c-rP
	632 (2)		628 (3)		628 (3)		632 (3)	cyclic ester ring def
671 (2)	674 (2)	667 (2, b)	673 (2)	660 (2, b)	670 (2, b)	661 (2, b)	670 (2, b)	G ring str, cyclic ester ring str
690 (s)	695 (s)		689 (s)					G ring (8-CH form)
715 (0)		712 (0)				715 (0, s)		5′-rP
				738 (0)	735 (0)	735 (0, b)	730 (0)	G ring + 8-CD def
	773 (1, b)		772 (1, b)		772 (1, b)		772 (1, b)	sym OPO str
788 (s)						790 (0)		ribose ring str
	810 (0)		800 (0)		800 (s)		805 (0)	antisym OPO str
814 (1, b)		806 (1, b)		802 (1, b)		810 (1, b)		5′-rP, PO str
		850 (0)		855 (0)				ribose ring str
	861 (1)		861 (1)		861 (1)		861 (2)	cyclic ester ring str
868 (1, b)						865 (1)		ribose ring str
880 (s)		880 (0)	887 (0)	880 (0)	880 (0)	890 (s)		5′-rP, c-rP, ribose ring
				935 (0)		930 (0)		5′-rP
	950 (0)						960 (0)	c-rP
978 (2)		978 (2)		978 (2)		978 (2)		sym PO_3^{2-} str
1000 (0)	1000 (0)	995 (0)	986 (0)	997 (s)	987 (1)	995 (0)	1000 (0)	c-rP, 5′-rP
	1030 (1)		1037 (1)		1044 (0)		1035 (1)	cyclic ester ring str
1050 (0)		1052 (0)		1055 (0)		1045 (1, b)		5′-CO str
	1083 (2)		1088 (2)		1088 (2)		1083 (3)	sym PO_2^- str
1085 (1, b)		1090 (0)		1090 (0)		1083 (1, b)		2′-CO str, ribose ring
1130 (0)	1115 (0)	1124 (0)	1123 (0)	1130 (0)	1126 (0)	1130 (0)	1118 (0)	ribose ring str
1180 (2)	1176 (2)	1176 (s)	1173 (s)	1190 (s)	1183 (s)	1182 (2)	1170 (s)	G ring external CN str
	1215 (0)						1195 (2, b)	G ring external CN str
1205 (0)	1230 (1)					1205 (1)	1222 (2)	antisym PO_2^- str; deg PO_3^{2-} str
1250 (0)	1260 (0)	1263 (s)	1262 (s)	1263 (s)	1259 (s)	1246 (0)	1250 (0)	G ring CN str + 8-CH def
				1307 (3)	1306 (3)	1305 (3)	1303 (3)	G ring CN str + 8-CD def
1324 (4)	1324 (4)	1324 (3)	1322 (3)					G ring CN str + 8-CH def
						1325 (0, s)	1325 (0, s)	G ring ND + 8-CD def
1365 (4)	1362 (4)	1353 (3)	1354 (3)	1350 (2)	1350 (3)	1357 (3)	1354 (3)	G ring CN str + NH def + 8-CH def
1415 (2, b)	1418 (2, b)	1385 (1) 1402 (1) 1424 (0)	1418 (1, b)	1397 (1, b)	1412 (1, b)	1408 (1, b)	1415 (1, b)	ribose CH + CH_2 def
				1458 (6)	1460 (6)	1464 (10)	1465 (10)	G ring CN str + 8-CD def
1487 (10)	1487 (10)	1480 (6)	1483 (6)					G ring CN str + 8-CH def
				1531 (1)	1530 (1)	1527 (2)	1530 (1)	G ring CN str + C=O str + 8-CD def
1538 (0)	1538 (0)	1538 (0)	1541 (0)					G ring CN str + C=O str + 8-CH def
1576 (6)	1576 (6)	1580 (10)	1580 (10)	1580 (10)	1580 (10)	1576 (6)	1576 (6)	G ring CN str + C=C str
1605 (0)	1605 (0)	1616 (0)		1616 (0)		1608 (0)	1605 (0)	G ring
		1667 (2)	1667 (2)	1665 (2)	1667 (2)			C=O str + C=C str
1680 (s)	1680 (s)					1675 (s)	1675 (s)	C=O str + C=C str + NH def
						2325 (1)	2325 (1)	8-CD str
		2890 (0)		2891 (0)				sym CH_2 str
			2915 (2)		2916 (2)			sym CH_2 str
		2950 (1)		2950 (1)				antisym CH_2 str
			2968 (1)		2968 (1)			antisym CH_2 str
			3006 (1)		3006 (1)			CH str
		3126 (0)	3126 (0)					8-CH str

[a] Structures I, II, III, and IV and other nomenclature are defined in the text. Abbreviations: G, guanine; 5′-rP, ribose 5′-phosphate moiety; c-rP, ribose 3′:5′-phosphate moiety; def, deformation; str, stretching; sym, symmetric; antisym, antisymmetric; s, shoulder; b, broad. Raman frequencies are accurate to ±2 cm^{-1} for lines of intensity 2 or greater and to ±4 cm^{-1} for other lines. Intensities (in parentheses) are given on an arbitrary 0 to 10 scale, with 10 assigned to the most intense line of each column.

of Table I are in complete agreement. Accordingly, the Raman line at 1665 cm^{-1} is assigned predominantly to C=O stretching and the line at 1578 cm^{-1} to C(4)=C(5) stretching. The latter is the only Raman frequency which is virtually unchanged for all derivatives of Table I. Moreover, a line of the same frequency is found also in the Raman spectra of adenine derivatives (Thomas & Livramento, 1975), as well as in the infrared spectra of both guanine and adenine derivatives. The intensity of the Raman line at 1578 cm^{-1}, like the infrared band, is usually sensitive to hydrogen-bonding and base-stacking interactions of the purines (Small & Peticolas, 1971; Thomas & Kyogoku, 1977; Howard et al., 1977). The present data support the conclusion that the normal coordinated for this frequency involves neither the N(1) nor the C(8) ring position (Tsuboi et al., 1973).

The cyclic nucleotide has Raman lines at 460, 570, 630, 665, 772, 805, 861, and 1040 cm^{-1} (phosphate–ester ring skeleton), at 1085 and 1225 cm^{-1} (PO_2^- group), and at 1414, 2916, 2968, and 3006 cm^{-1} (CH deformation and stretching vibrations). All of these frequencies occur also for cAMP. Raman lines of cGMP which are present only for H_2O solutions and therefore which probably involve the 2′-COH group occur at 625 and 955 cm^{-1}. The latter is shifted to 885 cm^{-1} for 2′-COD forms of cGMP. Vibrational assignments of the cyclic phosphate ester group, viz., symmetric OPO stretching, antisymmetric OPO stretching, symmetric PO_2^- stretching, and antisymmetric PO_2^- stretching, are consistent with the model compound studies of Forrest & Lord (1977).

Table II: Pseudo-First-Order Rate Constants[a] and Arrhenius Parameters[b] for 8-CH Exchange in 5′-rGMP and cGMP

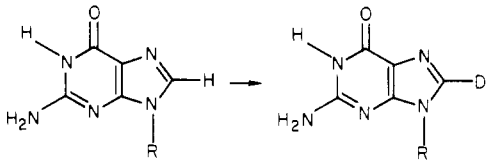
temp (°C)	5′-rGMP (h^{-1})	cGMP (h^{-1})
80	0.527	0.590
70	0.201	0.241
60	0.0694	0.0962
50	0.0196	0.0330
40	0.00610	0.0102
30	0.00171	0.00332
E_a (kcal/mol)	24.6 ± 0.6	22.2 ± 0.6
A (h^{-1})	8.84×10^{14}	3.33×10^{13}

[a] Reproducibility is within ±5% of the mean values cited from two or more determinations. [b] From least-squares curves of Figure 5.

We note that cGMP, unlike cAMP, contains no Raman line near 836 cm^{-1}. Therefore, our earlier assignment of this frequency in cAMP to the cyclic phosphate ester (Thomas & Livramento, 1975) is revised. Forrest & Lord (1977) also report no Raman line or infrared band at ~836 cm^{-1} for cyclic phosphate esters.

In 5′-rGMP, as other 5′ mononucleotides, Raman lines of the furanose ring are weak and broad. Few are easily assigned to specific group frequencies (Lord & Thomas, 1967; Small & Peticolas, 1971). The line near 800–815 cm^{-1} is likely due to the 5′-ester OP stretching and the line near 1050 cm^{-1} to 5′-CO stretching. The line near 865 cm^{-1}, which is clearly eliminated by deuteration of the 2′-COH and 3′-COH groups, probably involves a coupled furanose ring and an external CO stretching vibration. The symmetric and degenerate PO_3^{2-} stretching vibrations are assigned, respectively, at 978 (in all forms of 5′-rGMP) and 1205 (resolved only in H_2O solutions) cm^{-1}. CH deformations of ribose give rise to weak Raman lines in the interval 1380–1425 cm^{-1}, but the envelope is poorly resolved. CH stretching modes of the 5′-ribosyl ester occur at 2890 and 2950 cm^{-1}.

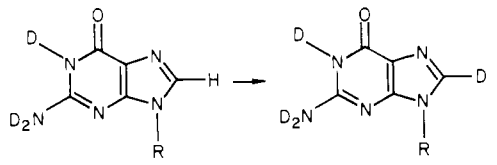
Spectral Effects Resulting from Isotopic Hydrogen Exchange of the C(8) Position in Guanine. The spectral changes resulting from replacement of hydrogen with deuterium at the C(8) position of each guanine nucleotide are seen by comparing the Raman frequencies of structures I and IV (H_2O solutions) or structures II and III (D_2O solutions) for that nucleotide in Table I. Thus, the substitution I → IV shifts



I IV

the prominent ring frequencies at 1538, 1487, 1365, and 1324 cm^{-1} to 1527, 1464, 1357, and 1305 cm^{-1}, respectively. At the same time, the doublet at 690 and 670 cm^{-1}, corresponding to I, is replaced by a single line near 660 cm^{-1}. IV but not I gives a ring frequency near 735 cm^{-1} which is clearly discernible in Figure 3. All of the Raman frequencies cited above are thus due to ring vibrations which are coupled with the 8-CH (or 8-CD) deformation of structure I (or IV).

The substitution II → III shifts the prominent ring modes



II II

at 1538, 1480, 1353, 1324, and 667 cm^{-1} to 1531, 1458, 1350, 1307, and 660 cm^{-1}, respectively. Again, these results indicate significant coupling of guanine ring stretching and 8-CH (or 8-CD) deformation vibrations. Deuteration of the 8-CH group also eliminates the Raman line at 3126 cm^{-1} due to CH stretching and introduces a new Raman line at 2325 cm^{-1} due to CD stretching (isotope shift factor 1.34).

As in the case of adenine derivatives, the concentrations of 8-CH and 8-CD forms of the purine ring in an isotopic mixture are best measured by the intensities of the corresponding Raman lines near 1480 and 1460 cm^{-1}, respectively (Livramento & Thomas, 1974; Thomas & Livramento, 1975).

Kinetics of Exchange in 5′-rGMP and cGMP. Figure 4 illustrates the pseudo-first-order kinetics governing deuterium exchange of the 8-CH group in 5′-rGMP and cGMP. Mean values of k_ψ for two or more independent determinations at each of several temperatures in the range 30–80 °C are given in Table II. Reproducibility is within 5% in each case.

Semilogarithmic plots of k_ψ against reciprocal temperature are given in Figure 5 and yield the Arrhenius parameters which are included in Table II. Figure 5 also contains for comparison similar plots for the adenine nucleotides 5′-rAMP and cAMP (Thomas & Livramento, 1975). The results indicate that ln k_ψ is a strictly linear function of $1/T$ for both 5′-rGMP and cGMP in the temperature range 30–80 °C. Only cAMP among the four prime nucleotides exhibits a significant deviation from linearity (Figure 5) by virtue of a more rapid than expected rate of exchange at lower temperatures. This anomalous result has been discussed previously (Thomas & Livramento, 1975).

Other interesting points of comparison between the adenine and guanine nucleotides are the following. (1) In the neighborhood of 30 °C, each cyclic nucleotide exchanges about twice as fast as its corresponding 5′ nucleotide. The gap narrows for each purine nucleotide pair as the temperature is increased. (2) 5′-rGMP exchanges its C(8) proton 2.5 times faster than 5′-rAMP throughout the temperature range studied. (Note the nearly parallel plots for 5′-GMP and 5′-rAMP in Figure 5, corresponding to the essentially identical activation energies of 24.6 ± 0.6 and 24.2 ± 0.6 kcal/mol, respectively.) (3) cGMP exchanges roughly 3 times faster than cAMP.

The present results are generally consistent with those obtained in tritiation studies of various purines (Tomasz et al., 1972; Elvidge et al., 1974; Gamble et al., 1976). In particular, Tomasz et al. (1972) report k_ψ = 0.0042 for guanosine at 37 °C, whereas Figure 5 yields k_ψ = 0.0044 for 5′-rGMP at 37 °C. Further, Gamble et al. (1976) have reported a twofold faster rate of tritiation in 5′-rGMP than in 5′-rAMP, consistent with the relative deuterium exchange rates. The only noteworthy discrepancy between previously reported results and those obtained here is the fact that specific rate constants cited by Maslova et al. (1969) and Gamble (1974) for 5′-rAMP and 5′-rGMP are uniformly lower (by a factor of ~2.1) than those reported here and elsewhere (Thomas & Livramento, 1975; Tomasz et al., 1972). This discrepancy cannot be due to an effect of pH on the mechanism since all experiments in question were carried out at a sufficiently high pH that the mechanism and exchange rate should not be affected (Tomasz et al., 1972).

It is possible that molecular interactions between guanine residues, such as are expected to be prevalent at pD 5 (Gellert et al., 1962) or at pD 7 and low temperature (Miles & Frazier, 1972), may account in a small part for the different rate constants obtained. It seems more likely to us, however, that

the different exchange rates for tritiation and deuteration of purine nucleotides are due to a combination of kinetic isotope effects and solvent isotope effects (Wolfsberg, 1969).

In comparing rates of deuteration and tritiation of a given purine ring, we accept the mechanism proposed by Tomasz et al. (1972), according to which the rate-determining step is solvent abstraction of the 8-CH proton from an "ylide intermediate". If this mechanism is correct, as all available evidence seems to indicate, then the rate of 8-CH exchange should not be affected appreciably by the isotopic mass of the group which is replacing the departing proton. Deuteration and tritiation of 8-CH should therefore yield essentially identical exchange rates if all other factors remain unchanged. Unfortunately, however, other factors do change. The deuteration experiments are carried out in pure D_2O solution, whereas the tritiation experiments were carried out in a solvent mixture of H_2O (80%) and T_2O (20%).

On the other hand, we may anticipate that the reverse or "back-exchange" reaction, i.e., 8-CD → 8-CH, could yield a significantly different rate constant than the forward exchange reaction (primary kinetic isotope effect). We are presently investigating this prospect by monitoring with Raman spectroscopy the back-exchange rates of purine nucleotides. Preliminary results indicate that reverse exchange is indeed slower than forward exchange in 5′-rGMP. A more detailed discussion of the comparative rates of forward and reverse exchange reactions will be published elsewhere (M. J. Lane and G. J. Thomas, Jr., unpublished experiments).

Conclusions

Exchange of hydrogen by deuterium at the C(8) position in guanine nucleotides is about 2.5 times faster than the corresponding exchange in adenine nucleotides. This means that in an equimolar mixture of adenine and guanine nucleotides ~82% of the guanine nucleotides would be exchanged in the time required to exchange only 50% of the adenine nucleotides. The advantage of G over A as regards 8-CH exchange is maintained at all temperatures in the range 30–80 °C.

Each cyclic purine nucleotide also exchanges more rapidly than its corresponding 5′ nucleotide provided the temperature is sufficiently low. Our results indicate that cGMP and 5′-rGMP would have identical exchange rates at 93 °C, whereas cAMP and 5′-rAMP have identical exchange rates at 80 °C (Thomas & Livramento, 1975). At 30 °C, each cyclic nucleotide exchanges about 1.9 times faster than its corresponding 5′ nucleotide.

The rate of isotope exchange at the C(8) position of a purine is known to be sensitive to the local structure around the base (Gamble et al., 1976). Factors which might be expected to play a role in influencing the exchange rate are the extent of base stacking, the dihedral angle between the plane defined by the purine ring and the plane of the N(9)–C(1)–C(2′) linkages, the electron charge distribution in the purine ring, and so forth. Unfortunately, there exists neither a theoretical nor an empirical basis for correlating the first-order rate constant or its temperature dependence with specific structural properties of the nucleotides. The present data do suggest, however, that the enhanced lability of the 8-CH bond in guanine nucleotides as compared with adenine nucleotides cannot be due solely to intermolecular interactions in one or the other type of purine nucleotide, because such interactions should diminish with increasing temperature. In other words, the 8-CH groups of guanine and adenine rings more probably exchange differently as a consequence of their different electronic structures. The electron-withdrawing effect of the conjugated 6-C=O group of guanine may well account for its more labile 8-CH bond. Indeed, the mechanism for exchange proposed by Tomasz et al. (1972) requires N(7) protonation as a first step. Since the pK governing N(7) protonation is greater for guanine than for adenine, the higher rate constants observed for guanine nucleotides are entirely consistent with the proposed mechanism.

Conversely, the fact that a difference in the rate of 8-CH exchange between 5′ and cyclic nucleotides of a given purine is favored by lower temperature suggests that intermolecular or intramolecular interactions are responsible. As shown by Gamble et al. (1976), the exchange rate of a purine is retarded by its participation in associative interactions such as base-stacking or base-pairing interactions of nucleic acid helixes. The present results could best be explained within such a framework by assuming that intermolecular interactions in 5′-rGMP (or 5′-rAMP) retard the rate of 8-CH exchange as compared with cGMP (or cAMP) and that such intermolecular interactions and attendant retardation of exchange are eliminated with increasing temperature. This explanation would also account for the fact that aqueous 5′-rGMP must be heated to a higher temperature than aqueous 5′-rAMP in order to effectively eliminate the retardation of 8-CH exchange vis-à-vis the corresponding cyclic nucleotide.

Acknowledgments

M.J.L. is indebted to Betty Prescott for expert technical assistance and helpful advice in all phases of the experimental work.

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Complete Assignment of Carbon Signals in a Stereospecific Peptide via Selective and Single Off-Resonance Proton Decoupling Experiments. Analysis of the Carbon-13 Nuclear Magnetic Resonance Spectrum of Alumichrome at 67.88 MHz†

Antonio De Marco and Miguel Llinás*

ABSTRACT: Polypeptides and proteins in native conformation exhibit ^{13}C NMR spectra which are highly nondegenerate. Assignment of resonances to carbons in particular residues is hence a prerequisite for a structural analysis of the spectroscopic data. For nonprotonated carbonyl carbons, the assignment can be achieved by selective $\{^1H^{\alpha}\}^{13}C'$ ^2J decoupling. Using this method, we have assigned the Orn^1 and Gly^2 carbonyl resonances in alumichrome at 67.9 MHz. We show that a single off-resonance experiment with the decoupling frequency centered in the aliphatic proton spectrum is sufficient to assign unequivocally all the protonated carbon resonances via analysis of the reduced ^1J heteronuclear splittings. Alumichrome thus becomes the first complex polypeptide spin system whose ^1H, ^{15}N, and now ^{13}C nuclear resonances have been fully identified to date. ^{13}C chemical shifts and ^1H–^{13}C spin–spin couplings are discussed in terms of structural strain leading to specific orbital hybridizations and on the basis of polarization effects due to electron density shifts toward hydrogen-bonding and metal-binding sites. A number of $^3J(^{13}C\text{–}C\text{–}C\text{–}^1H)$ coupling constants measured on selected multiplets after resolution enhancement were used to derive the χ-related Karplus relationship

$$^3J(\theta) = (10.2 \cos^2 \theta - 1.3 \cos \theta + 0.2) \text{ Hz}$$

The assignment of signals in NMR spectra is a crucial step for any conformational interpretation of data. Polypeptides in native nonrandom conformations exhibit ^{13}C NMR spectra that reflect the particular structures (Komoroski et al., 1976; Llinás et al., 1976a). This means that ^{13}C spectra cannot be accounted for in terms of a simple addition of the individual residue subspectra; rather, the spectra show a complex dependence on the structural electronic microenvironment at each particular site. In view of the lack of an adequate theory of ^{13}C chemical shifts, assignment of resonances often is a major problem which can be partly overcome by laborious selective isotopic enrichment (Grathwohl et al., 1973; Sogn et al., 1974), by specific chemical modification (Norton & Allerhand, 1976; Dill & Allerhand, 1977), or, as with ^1H NMR (Llinás et al., 1972), by comparative spectroscopy on species-related polypeptides (Packer et al., 1975; Oldfield et al., 1975; Wilbur & Allerhand, 1977). By use of the latter technique on various isomorphic alumichrome homologues which differ in single residue substitutions at sites 2 and 3 (Figure 1), α-carbon resonances of Gly^1, Gly^2, and Gly^3 could be confidently assigned, while the invariant, nonsubstituted ornithyl C^{α} resonances were only tentatively identified on the basis of weak, nearest-neighbor perturbations and correlations with $^1H^{\alpha}$ chemical shifts (Llinás et al., 1976a, 1977a). Thus, although the comparative data enabled classification of groups of resonances in terms of various carbon types (e.g., ornithyl C^{α}'s, C^{β} + C^{γ}'s, C^{δ}'s, etc.), most of the carbon signals remained unassigned. This reflects a weakness of the approach in that critical residues tend to be evolutionarily invariant and thus difficult to identify by comparative spectroscopy.

In the past, we have developed a technique which enables unequivocal assignment of ^{13}C resonances by sequential $^1H\{^{15}N\}$ and $^{13}C\{^{15}N\}$ experiments on uniformly ^{15}N-enriched peptides (Llinás et al., 1977b). This led to a total solution of the difficult problem of assigning resolved carbonyl resonances in alumichrome (Figure 1). Such heteronuclear decoupling experiments were feasible only because the amide proton spectrum had previously been characterized (Llinás et al., 1972); this provided a starting point for identifying ^1J-coupled ^{15}N resonances (Llinás et al., 1976b) and consequently, by an identical procedure, the connected carbonyl ^{13}C signals. Hence, in principle at least, ^{13}C NMR signals can be identified if the spectrum of other spin–spin-coupled heteronuclei has previously been characterized.

The assignment of ^{13}C spectra of molecules of a certain complexity by single-frequency ^1H decoupling can be nontrivial when the proton spectrum is strongly coupled and resonances overlap extensively. Unambiguous interpretations are hard to achieve because multicoupled spin systems often exhibit second-order effects. Under such circumstances, selective decoupling is difficult at low magnetic fields and tedious and intricate at high field strengths. In contrast, off-resonance irradiation causes a reduction of the splittings due to heteronuclear couplings (Ernst, 1966), which are relatively simple to detect and measure. The technique, increasingly applied to simple organic compounds (Tanabe et al., 1971), can be

† From the Istituto di Chimica delle Macromolecole, Consiglio Nazionale delle Ricerche, 20133 Milano, Italy (A.DeM.), and the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213 (M.Ll.). *Received March 30, 1979.* This research was supported by the donors of the Petroleum Research Fund, administered by the American Chemical Society, and by the Italian National Research Council.

0006-2960/79/0418-3846$01.00/0